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(54) Title: USE OF NERVE GROWTH FACTOR FOR THERAPY OF INTRAOCULAR TISSUE PATHOLOGIES		
(57) Abstract Nerve Growth Factor (NGF), in the form of a preparation to be administered over ocular surface, is suggested as being suitable for therapy and/or prophylaxis of intraocular tissue pathologies, with particular reference to sclera, ciliary body, crystalline lens, retina, optic nerve, vitreous body and choroidea affections. When administered in the form of external ophthalmic preparation, for example as collyrium or ointment, NGF is capable to go through ocular tissues and it has been found out that it shows a therapeutic activity not only against retina and optic nerve pathologies but also against affections involving the above reported internal structures of the eye.		

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USE OF NERVE GROWTH FACTOR FOR THERAPY OF INTRAOCULAR
TISSUE PATHOLOGIES

The present invention relates to the use of nerve
5 growth factor for the therapy of intraocular tissue
pathologies. More particularly, the invention relates to
the use of the neurotrophin, named nerve growth factor
(NGF), for the therapeutic treatment of the eye internal
structures, as sclera, choroidea, ciliary bodies,
10 crystalline lens, vitreous body, retina and optic nerve,
by a topical administration over the ocular surface, i.e.
as collyrium or ophthalmic ointment.

The nerve growth factor (NGF) is the chief molecule
of a complex neurotrophin family, and is well known for
15 its trophic, tropic and differentiating activity on
cholinergic neurons of the central nervous system and on
the sympathetic peripheral system. NGF is produced by
various mammalian tissues, included humans, and is
released in the circulatory flow in greater amounts
20 during the growth and differentiation of the nervous
system. Biological, biochemical and molecular studies
carried out on *in vitro* cellular systems have pointed out
high sequence homology between murine and human NGF.
Furthermore, in humans and other mammals NGF is
25 normally contained both in the cerebrospinalis liquor and
blood flow at concentrations of about 10-15 pg/ml. The
value increases during some inflammatory pathologies
(autoimmune and allergic diseases, etc.), whereas
decreases in others (diabetes).

30 NGF has been discovered by Prof. Rita Levi-
Montalcini, at the Zoology Institute of St. Louis

Washington University (Levi-Montalcini R., Harvey Lect., 60:217, 1966), and its discovery represented a remarkable step for studying mechanisms of growth and differentiation of nerve cell, being able to affect the development and preservation of the biological functions and the regeneration of the neurons. In 1986 the Nobel Prize for Medicine and Physiology was assigned to Prof. R. Levi-Montalcini for the discovery and characterization of biological function both in peripheral and central nervous system of this molecule.

Various experimental studies both *in vitro* and *in vivo* have demonstrated the NGF physiopathological importance to prevent neuron damages of surgical, chemical, mechanical and ischemic origin, allowing it to be used as ideal compound for the therapy of various pathologies affecting both the peripheral and central nervous systems (Hefti F., J. Neurobiol., 25:1418, 1994; J. Fricker, Lancet, 349:480, 1997). In fact some years ago clinical tests have been carried out on subjects affected by Parkinson's Disease and Alzheimer's Disease by intracerebral administration of murine NGF (see, for example, Olson L. et al., J. Neural Trans.: Parkinson's Disease and Dementia Section, 4:79, 1992). Results of these experiments confirmed observations obtained from animal models and pointed out the absence of possible side effects following the administration of murine NGF. This behaviour has been confirmed more recently for recombinant human NGF (Petty B.G. et al., Annals of Neurobiology, 36:244-246, 1994).

Studies referring to the characterization of biological, biochemical, molecular, pre-clinical and clinical effects almost exclusively have been carried out

using NGF isolated from submandibular glands of adult rodents; therefore available data concern mostly murine NGF. Biochemical properties of the latter, particularly, have been described in a study published in 1968 (Levi-Montalcini R. and Angeletti P.U., Physiological Reviews, 48:534, 1968).

NGF contained in murine salivary glands is a 140 kdalton molecular complex, the sedimentation coefficient thereof being 7S, and it is constituted by three sub-units, α , β and γ , the second of which represents the actual active form. The latter, called β NGF, whose sedimentation coefficient is 2.5S, is usually extracted and purified according to three not very different techniques (Bocchini V., Angeletti P.U., Biochemistry, 64; 787-793, 1969; Varon S. et al., Methods in Neurochemistry, 203-229, 1972; Mobley W.C. et al., Molecular Brain Research, 387:53-62, 1986).

The so obtained β NGF is a dimer of ~ 13.000 dalton, constituted by two identical chains of 118 amino acids. Each chain is stabilised by three disulphide bridges, while not covalent bonds assure the stabilisation of the dimeric structure. The molecule is very stable and is soluble in almost all solvents, both aqueous and oily, maintaining unchanged its biochemical characteristics and biological activity. Further details about the structure, physical and biochemical properties of the molecule are reported in Green, L.A. and Shorter, E.M., Ann. Rev. Neurosci., 3:353, 1980.

Recently the structure of β NGF has been further disclosed by means of crystallographic analysis. The analysis pointed out the presence of three anti-parallel

filament pairs, having a β -type secondary structure, forming a flat surface along which the two chains join together resulting in the active dimer. On these β NGF chains the presence of four "loop" regions has been
5 showed, wherein are included many variable amino acids probably responsible for receptor recognition specificity.

The NGF biological effect is mediated by two receptors present on the corresponding target cells. The
10 existence of various antibodies that selectively inhibit the NGF biological effect has allowed an accurate characterization and modulation of the activity thereof, both in cellular systems and *in vivo*.

More recently human NGF has been synthesized using
15 genetic engineering techniques (Iwane et al., Biochem. Biophys. Res. Commun., 171:116, 1990) and small amounts of human NGF are commercially available too. However the author of the invention discovered that the biological activity of human NGF is very low when compared to murine
20 NGF. Furthermore it is to be pointed out that almost all of data available concerning human NGF, both *in vivo* and *in vitro*, have been obtained using murine NGF and undesirable side-effects resulting from murine origin of molecule have never recognised.

25 Studies carried out since 90's using animal models suggested a possible NGF involvement in ocular pathologies. Apart of some patent publications wherein NGF is not the object of actual experimental results, but is only mentioned together with other known growth
30 factors (on the basis of the unverified assumption that it belongs to an homogeneous class of molecules having equivalent characteristics and biological activities),

and apart of the PCT patent application No. WO98/48002, under the Applicant's name, wherein the use of NGF in the therapy for cornea and conjunctiva pathologies is suggested (discussed in detail below), the scientific reports published in the ophtalmic field exclusively refer to the use of NGF for retina and optic nerve affections.

Particularly it has been reported that the intraocular NGF administration to animal models is effective for enhancing the survival of retinal ganglion cells following acute retina ischemia (Siliprandi R. et al., *Inv. Ophthalmol. Vis. Sci.*, 34:3232, 1993) and optic nerve trans-cutting (Carmignoto G. et al., *J. Neurosci.*, 9:1263, 1989). More recently the NGF administration by intra-vitreous or also retro-bulbar injections proved to be effective for the mouse retinal degeneration model, which is similar to human pigmentary retinopathy (Lambiase A. and Aloe L., *Graefe's Arch. Clin. Exp. Ophthalmol.*, 234:S96-S100, 1996), and for the rabbit retinal damage model resulting from ocular hypertension (Lambiase A. et al., *Graefe's Arch. Clin. Exp. Ophthalmol.*, 235:780-785, 1997).

Such experimental studies showed that the local NGF administration is effective for preventing or at least delaying the death of retinal ganglion cells and photoreceptors resulting from above said pathologies. In addition side effects during animal treatments have not been reported. However it is to be pointed out that in all the publications above reported, NGF is administered to the ocular tissue by intra-vitreous or also retro-bulbar injection.

The PCT patent application No. WO98/48002 up to now is the only document wherein the use of NGF as external ophthalmic application, for example in the form of collyrium or ointment, is described. Experimental work therein reported proves that topically administered NGF is suitable for a successful treatment of ocular surface pathologies (cornea and conjunctiva) both of acquired and congenital type and, particularly, of various dystrophic or neurodystrophic pathologies for which therapeutic treatments did not exist previously. The discovery of the presence of NGF and of its high affinity receptor (TrkA, tyrosinkinase A), by immunohystochemical techniques, was the condition for such innovative result. Evidently the expression of the NGF high affinity receptor is an essential prerequisite for NGF to exert its therapeutic activity.

During the studies of the instant invention, always by both immunohystochemical and immunofluorescence techniques (Lambiase et al., J. Allergy Clin. Immunol., 100:408-414, 1997) and biomolecular techniques as well for the *in situ* identification of the NGF mRNA (Micera A. et al., Archives Italiennes de Biologie, 133:131-142, 1995), it has been pointed out that any cell contained in sclera, crystalline anterior capsule, ciliary body epithelium, optic nerve fibers, retinal ganglion cells, retinal pigmented epithelium cells and some choroidea cells not only express the receptor having high affinity for NGF but are also able to produce this neurotrophin (not yet published data). The experimental data result in various implications. On the one hand NGF, released from cells of various ocular tissues, should exhibit a trophic and physiopathological activity in all the ocular

regenerative mechanisms; on the other hand various pathologies of trophic, degenerative or immune type should recognise the failed release of NGF as fundamental etiologic chance.

5 Furthermore, because the effects observed after the administration of exogenous NGF are present at almost physiological concentrations (in the order of about a few micrograms), it is conceivable that in some ocular affections the reduction of local NGF levels under the
10 threshold value suitable to assure the tissue integrity can be a possible physiopathogenetic mechanism. Such a pathogenetic hypothesis is confirmed by the effects derived from NGF deprivation, both *in vivo* and *in vitro*, that induces the death of various cell population and the
15 exacerbation of tissue damages of chemical, physical, infective or degenerative type (Aloe L., *Int. J. Devl. Neuroscience*, volume 5(4), 1987; Lambiase A. and Aloe L., above reported; Lambiase et al., *Graefe's Arch. Clin. Exp. Ophthalmol.*, 1997, above reported).

20 Although the above results allow to hypothesise a therapeutic activity of NGF also for ocular structures and tissues different than those already reported in literature, and specifically for sclera, ciliary body, crystalline, vitreous body and choroidea, there is the
25 problem for an easy administration of the active principle to involved tissues. Contrary to the case considered in the PCT patent application No. WO98/48002, referring to cornea and conjunctiva pathologies, herein tissues within bulb of eye are involved.

30 The possibility of an external topical administration for an ophthalmic therapeutic agent, i.e. in the form of collyrium or ointment, represents a

remarkable benefit in comparison with the administration through parenteral topical, retrobulbar or intravitreal injection routes. In fact the use of these latter techniques involves the risk for various complications, reported in literature, as the ocular bulb perforation, infections, haemorrhages and lesions of anatomical structures during injection. Such complications can occur also more frequently during the treatment of chronic pathologies, and can lead to the unfeasibility of the therapy due to the inversion of risk/benefit ratio.

The author has surprisingly found that by administration of NGF in the form of collyrium, an increase of such a neurotrophin levels in all ocular tissues, including those into the ocular bulb, is obtained. As it will be illustrated in detail in the following experimental report, the passage of NGF from the ocular surface, where it is administered, to internal ocular tissues, has been showed using both an autoradiographic method (Levi-Montalcini, R and Aloe L., Proc. Natl. Sci. USA 82:7111-7115, 1985), and an immunoenzymatic assay (Bracci-Laudiero, L. et al., Neurosci. Lett., 147:9-12, 1992). The application of the latter method on rabbits treated by conjunctival instillation of a NGF-containing saline solution has caused, one hour after the administration, an increase of NGF concentration in all the examined ocular tissues. The NGF level is reduced to initial levels after 6-8 hours. This effect allows NGF to express its therapeutic activity also in not directly involved tissues by a superficial administration. This aspect is innovative not only with reference to the ophthalmic pathologies for which till now the NGF therapeutic activity was not even

conceivable, but also for retina and optic nerve pathologies, wherein the NGF possible activity has been already reported, but it was not yet available a drug administration in a ready and safe way without risks and drawbacks for the patient.

Therefore it is a specific object of the present invention, according to a first aspect thereof, the use of nerve growth factor (NGF) for the production of an ophthalmic preparation to be administered over the ocular surface for the therapy and/or prophylaxis of intraocular tissue pathologies. Specifically said NGF containing ophthalmic preparation is in the form of solution or suspension (collyrium), ointment, gel or liniment together with a pharmaceutically acceptable, eye tolerated and compatible with active principle ophthalmic carrier. It is also possible to conceive particular routes for ophthalmic administration for delayed release, as ocular erodible inserts, or polymeric membrane "reservoir" systems to be located in the conjunctiva sac. Alternatively NGF, or a preparation containing it, can be added to a local bandage together with a therapeutic contact lens.

As already pointed out said ophthalmic preparation is suitable for the therapy and/or prophylaxis of sclera, ciliary body, crystalline lens, retina, optic nerve, vitreous body and choroidea pathologies, said affections having trophic, post-traumatic, infective, post-surgical, autoimmune, dystrophic, degenerative, post-inflammatory and laser treatment origin. As it will be demonstrated by experimental data below reported, the NGF external topical administration proved, among other things, to be able to repair sclera lesions of traumatic or immune

origin, to cause an increase of aqueous humour production, restoring the intraocular pressure in pathologies characterised by hypotonia and resulting in bulbar phthisis and to prevent and delay the formation and progression of crystalline lens opacity (cataract).
5 As to retinal pathologies, the NGF administration by application over ocular surface induces an increase of nervous fiber thickness, a survival of retina ganglion cells, photoreceptors, pigmented epithelium during
10 degenerative, ischemic, traumatic pathologies and when damages from ocular hypertonia are present. As to optic nerve the effects obtained are an improvement of visual evoked potentials (PEV), visual field and survival of nervous fibers when traumatic, ischemic, pressor and
15 degenerative pathologies occur. Finally as to choroidea the NGF administration by external ophthalmic application causes a reduction of choroidea inflammatory processes and reduces the number of mobile vitreous bodies. It is to be pointed out that many of these disorders are hardly
20 therapeutically treated, or they lack of an effective treatment.

The possibility that nerve growth factor could exhibit a biological activity on internal tissues of ocular bulb following an external local administration
25 was hardly predictable mainly considering that, as before pointed out, NGF is a quite big molecule (26.800 dalton) with a complex structure. In order that a molecule can exert its activity on deep ocular tissues, it is necessary that, once it has been instilled over the eye
30 surface, the molecule pass through the lacrimal layer, cornea, aqueous humour and vitreous body so to be distributed within all the tissues. According to the

current practice such molecules (particularly antibiotics or cortisone molecules) which are able to reach the crystalline lens, vitreous body and retina at therapeutically effective concentrations are not
5 available. For the above reasons in all the known studies on the utilisation of NGF for ocular pathologies, only the intraocular administration route was used.

In effect NGF, although has a complex structure and high molecular weight, includes both hydrophilic and
10 hydrophobic groups which allow it to pass through the homologous (lipid and hydrophilic) anatomical barriers. Furthermore it is a basic characteristic of NGF that once it has reached target organs, also at very low but yet biologically active concentrations, it is able to
15 stimulate tissue to produce endogenously the NGF. The presence of an endogenous produced NGF is clearly suggested by experimental results concerning the NGF passage through tissues. These results furthermore show that a concentration gradient is not maintained from the
20 external surface to deeper eye tissues, as it would be conceivable in the presence of a simple diffusion mechanism through the tissues.

In order to carry out the preparation according to the present invention suitable procedures for the NGF
25 extraction and purification are reported in the previously cited references. The technique according to Bocchini and Angeletti, herein briefly reported, has been used for the experiments of the present invention. Submandibular glands of adult male mice are collected in
30 a sterile way and tissues thereof are homogenised, centrifuged and dialysed; then the obtained suspension is passed through subsequent cellulose columns, whereon NGF

is adsorbed. Following NGF is eluted with a buffer containing 0.4 M sodium chloride. The obtained samples are analysed spectrophotometrically at a 289nm wavelength to identify the NGF containing fractions. These fractions
5 are dialysed and the NGF is lyophilised in a sterile way and stored at -20°C in freezer.

A medicament according to the invention suitable for administration onto the ocular surface preferably contains, alone or in association with one or more other
10 active principles, from 1 to 1000 µg/ml of NGF. In the case the product is in the form of an aqueous solution (collyrium), the concentration of NGF is preferably between 10 and 500 µg/ml. A specific formulation suitable in the form of collyrium contains, for example, 200 µg/ml
15 of NGF in physiological solution containing 0.9% of sodium chloride, or in balanced saline solution (BSS^R); in both circumstances the solution is isotonic with lacrima and therefore well tolerable by the eye. However it is also possible the use of hypotonic solutions.

20 The NGF contained in the saline solution can be present alone or in association with other biologically active molecules, and/or conjugated with carrier molecules (as, for example, transferrin). In order to further enhance its passage through ocular surface, other
25 excipients selected from those conventionally used according to pharmaceutical techniques, for example to buffer the solutions or suspensions, to stabilise the active principle and make the preparation well tolerable can be added. Specifically buffers should keep pH between
30 4 and 8. For example the above reported sodium chloride solution can be buffered using any of the buffers well known in the pharmaceutically field as suitable for

ophthalmic use, among which phosphate or trizma (tri-hydroxymethyl-aminomethane) buffers, so to have a physiological pH, i.e. 7.0-7.4, maintaining simultaneously a physiological osmolarity (295-305 mOsm/l).

The tolerability can be further enhanced using excipients like polysorbate 80 (or Tween 80), dextran, polyethylene glycol (for example PEG 400) and like. The formulation can contain also viscosity-enhancing agents like hyaluronic acid, methylcellulose, polyvinylalcohol, polyvinylpyrrolidone and others, in order to enhance the ocular bioavailability, stability and tolerability of the active principle. The ocular bioavailability of NGF can be further enhanced by using compounds that ameliorate the corneal permeation of the drug as, for example, dimethylsulfoxide, taurocholates, membrane phospholipids and various surfactant agents suitable for ophthalmic use. In addition to prevent contamination, a preservative agent having antimicrobial activity can be added to the formulation.

Agents like carboxymethylcellulose or like can be added to products to be administered in form of suspension. If it is desired to use the formulation in the form of ointment, gel or ophthalmic liniment, the NGF carrier could be polyethyleneglycol, polyacrylate, polyethyleneoxide, fatty acid and alcohol or lanolin, paraffin and similar products.

As already pointed out the therapeutic activity of nerve growth factor against ocular tissues other than superficial (cornea and conjunctiva), retina, optic nerve has been not previously disclosed neither when it is administrated by intraocular injection nor by

formulations in the form of collyrium or ointment. Therefore it is a further object of the invention the use of nerve growth factor (NGF) to produce an ophthalmic preparation for the therapy and/or prophylaxis of intraocular tissues pathologies, except retina and optic nerve pathologies, whatever the administration route is.

Again the concentration of NGF in the preparation is preferably between 1 and 1000 µg/ml of NGF and all the conventional formulation procedures well known in the field can be used and particularly those previously reported with reference to the ophthalmic formulations for external administration.

Some experimental results, obtained within the scope of the present invention, including clinical data concerning therapeutic applications on humans, are below reported merely for exemplary purposes.

Studies on the passage of NGF through ocular tissues

In a first set of tests to study the passage of NGF intraocularly from external surface over which it was administered, the above mentioned autoradiographic method has been used for a group of six rabbits. Each of the animals was administered with one collyrium drop (50 µl) containing 10 µg of I¹²⁵ labeled NGF (concentration: 200 µg/ml) by instillation in the conjunctiva fornix.

Murine NGF purified according to the previously described method and subsequently conjugated to Na-I¹²⁵ (Amersham Italia, IMS30, 1mCi) according to chloramine T method (Lapack PA. Exp. Neurol. 124:1620, 1993) has been used. The amount of labeled NGF has been determined by chromatography using a Sephadex G-25 column. The amount of the I¹²⁵ labeled product collectible by precipitation

was between 90% and 95%, showing that the most of the radioactive product was bonded to NGF. The specific activity of NGF-I¹²⁵ was between 1 and 1.5 Ci/ μ mol.

Two hours following the administration of the labeled NGF the animals were sacrificed and eyes enucleated and fixed in 4% paraformaldehyde over 48 hours. Then samples, after incubation in 30% sucrose over 24 hours, were cut with a cryostat to 15 μ m thick sections. Sections were mounted on histology gelatinous slides, immersed in photographic emulsion (Ilford K2) and incubated over 4 weeks at 4°C. Sections were successively dehydrated using ethanol, mounted on DPX after treatment with xylene and examined with Zeiss optical microscope.

This experiment showed that labeled NGF, after its administration over ocular surface, was able to penetrate into eye and bond with cells of various tissues contained in the posterior segment and crystalline lens inducing the expression of the specific receptor.

In a second set of tests, using above described immunoenzymatic method, the quantitative levels of NGF in various ocular tissues after the administration by instillation of a drop of murine NGF in the conjunctiva fornix were determined. In all 24 rabbits were used, six thereof were sacrificed immediately to determine initial values of NGF concentration in various ocular tissues. Remaining animals were sacrificed after 1 (6 rabbits), 2 (6 rabbits) and 8 hours (6 rabbits) following the administration of the collyrium.

In all the cases the eyes were enucleated and the different tissues (cornea, sclera, aqueous, iris, crystalline lens, retina, choroidea, optic nerve) were sectioned. The tissues were weighted, sonicated (using

Braun B Sonicator) in a buffered protein matrix containing protease inhibitors (extraction buffer). Thus obtained homogenate was centrifuged (x 10000 rpm for 20 minutes) and supernatant was used to determine the levels on NGF by immunoenzymatic method (ELISA). This technique is extremely sensitive and NGF specific and it is able to detect concentrations up to 5 pg/ml. Goat anti-NGF polyclonal antibody, diluted in 0.05 M carbonate buffer, pH 9.6, was used as first antibody. As control, for the determination of unspecific signal, purified goat immunoglobulins were used.

Solutions containing primary antibody and control immunoglobulins were plated in parallel on polystyrene 96 well plates. Then the plates were incubated for 12 hours at room temperature and following the unspecific sites were blocked using a solution containing carbonate buffer plus 1% BSA. Further to plate washings with 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 0.5% gelatine, and 0.1% Triton X-100, NGF samples and standard solutions were suitably diluted with 50 mM Tris-HCl, pH 7.2, 400 mM NaCl, 4 mM EDTA, 0.2 mM PMSE, 0.2 mM benzethonium chloride, 2 mM benzimidazole, 40 U/ml aprotinin, 0.05% sodium azide, 2 % BSA and 0.5 % gelatine. After triplicate distributions of standard solutions and samples of NGF in an amount of 50 μ m/well, plates were incubated with the secondary antibody: 4 mU/well of anti- β -galactosidase (Boehringer Mannheim, Germany) for 2 hours at 37°C. Then, after the washings, 100 μ l/well of a solution containing 4 mg of β -galactosyl-chlorophenol red (Boehringer Mannheim Germany)/ml of 100 mM HEPES, 150 mM NaCl, mM MgCl₂, 0.1% sodium azide and 1% BSA solution were distributed.

After the incubation of the chromogen for a period of two hours at 37°C the optical density at wavelength of 575 nm was determined using ELISA reader (Dynatech). The concentration values of NGF standards and samples were calculated after subtraction of background values due to unspecific bonds. Data reported as pg/ml or pg/g are referred to fresh weighted tissue. Results, resumed in the following Table 1, show that: after one hour from the collyrium administration in all the intraocular tissues the NGF concentration values are increased, these values are maintained high, although reduced, and after 8 hours they are again the same as the initial ones.

Table 1

NGF concentrations in various ocular tissues after NGF administration in the form of collyrium
(NGF pg/g of tissue)

HRS	SCLERA	CHOROIDEA	RETINA	OPTIC NERVE	CRYSTALLIN E LENS	VITREOUS BODY
0	100 ± 50	960 ± 400	83 ± 50	83 ± 50	100 ± 15	10 ± 4
1	1414 ± 30	2800 ± 700	484 ± 70	1195 ± 180	200 ± 30	73 ± 12
2	694 ± 150	1813 ± 900	322 ± 100	342 ± 115	150 ± 20	20 ± 5
3	200 ± 100	100 ± 500	150 ± 70	130 ± 100	110 ± 20	10 ± 5

Studies on the effect of NGF administration in the form of collyrium for sclera pathologies

Presently therapeutic treatments effective to induce reparations for both traumatic and immune or infective sclera lesions are not known. In the case of autoimmune pathologies the formation of malacic sclera

zones (scleromalacia) occurs which tend progressively to enlarge and become deeper with possible bulb perforation. Surgical treatment is the unique usable therapy and it includes the coating of damaged or malacic zone with a layer of human stored sclera or other biocompatible human tissues. However in the case of immune affections, recidivations of sclera pathology often occur.

In the studies in connection with the present invention the effect of external administration in the form of collyrium of murine NGF (2.5S), at a concentration of 250 µg/ml in balanced saline solution, was evaluated for 4 cases of sclera lesions, 2 of which post-traumatic and 2 scleromalacic by autoimmune diseases (reumatoid arthritis, AR and systemic lupus erythematosus, respectively). Therapeutic protocol included the daily instillation of one or two drops of preparation in the following way: during the first two days every two hours, six times a day up to the second day from the complete sclera reparation and four times a day during the following fifteen days. Therapy, once interrupted, should immediately again carried out if initial signals or symptoms of recidivations of sclera pathology are present.

All the patients within two weeks from the beginning of the treatment with NGF showed clear signals of recovery. None thereof showed occurrence of local or systemic side effects during or after the treatment. Obtained data are summarised in the following table.

Table 2
Effect of treatment with NGF in the form of collyrium for
sclera pathologies

Pat. No.	Pathology	Age years Sex	Occurrence	Extension	NGF Treatment	Outcome	Follow up
1	perforating trauma	35, F	4 days	4 mm	21 days	recover y	8 months
2	perforating trauma	42, M	5 days	6 mm	25 days	recover y	6 months
3	scleromacia in AR	55, F	30 days	5 mm	20 days	recover y	10 months
4	scleromacia in LES	42, M	25 days	4 mm	17 days	recover y	8 months

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Studies on the effect of NGF administration in the form of collyrium for the production of aqueous humour

Effect of topical administration of NGF on the production of aqueous humour was determined first on a set of 6 normal pressure rabbits. Using a tomography based method including a probe in anterior chamber of eye which is able to evaluate the modifications in the production of aqueous humour, it was recognised that the administration of NGF in the form of collyrium every two hours at a concentration of 200 µg/ml, in balanced saline solution, induces a five-fold increase in the production of aqueous humour. Such an increase is maintained during all the period of treatment.

On the base of the results obtained on animal model three patients with remarkable ocular hypotonia, in two of which following surgical treatments (2 eyes) and the other by relapsing chronic uveitis. Due to very low intraocular pressure values (< 4 mm Hg), rapidly medical

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conditions were degenerating to bulb phthisis. The therapeutic protocol included the instillation of one or two drops of NGF preparation (200 µg/ml) in balanced saline solution every two hours until a successful clinical outcome.

All the treated patients exhibited clear symptoms of recovery within two weeks from the beginning of NGF treatment, intraocular pressure values being again between 8 and 12 mm Hg within 4 weeks. None patient showed the occurrence of local or systemic side effects during the treatment or the following period. Obtained data are summarised in the following table.

Table 3

Effect of the administration of NGF in the form of collyrium on production of aqueous humour

Pat. No.	Pathology	Age years Sex	Occurrence	NGF Treatment	Outcome	Follow up
1	vitrectomy	40, M	30 days	21 days	9 mm Hg	7 months
2	vitrectomy	53, F	25 days	25 days	10 mm Hg	11 months
3	chronic uveitis	45, F	40 days	20 days	12 mm Hg	10 months

Studies on the effect of NGF treatment in the form of collyrium for the cataract prevention

Because it has been recognised that cells of crystalline lens capsule express the receptor with high affinity for NGF and simultaneously produce this neurotrophin, it was studied whether variations of local values of NGF resulted in formation of crystalline lens

opacity (cataract, a process usually related to senescence phenomena, diabetes, steroid treatment, traumas or physical stresses) and whether the topical administration of NGF could prevent the formation or progression thereof.

To demonstrate the activity of NGF firstly a model for in vitro formation of cataract was used. In the study 18 crystalline lenses from adult rats were collected and incubated in a xilose containing medium. Then 6 crystalline lenses were treated by the addition to the medium of amounts of murine NGF variable between 1 and 300 pg/ml, 6 crystalline lenses were treated by the addition of amounts of anti-NGF antibody between 500 and 1000 µg and the remaining were left untreated as control. After 48 hours from the beginning of the culture it was clear that 6 crystalline lenses treated with anti-NGF antibody exhibited almost full cataract, whereas 6 control crystalline lens exhibited cortical cataract with poor involvement of nucleus of crystalline lens. Remaining 6, treated with NGF, exhibited only rare opacity traces, the best response being obtained with NGF concentration of about 200 pg/ml in culture medium.

To confirm the in vivo NGF activity in preventing the cataract occurrence a cataractogenesis animal model involving a diet including 30% glycerol was used. All the animals (100%) subjected to this diet exhibit a cataract within 44° day. A group comprising ten animals was treated by three daily administrations of NGF in the form of collyrium at a concentration of 200 µg/ml in balanced saline solution, a second group again comprising ten animals was subjected to a treatment with anti-NGF antibodies injected in the anterior camera and the last

group of animals was treated with saline solution in drops and was used as control.

All the rats of the group treated with anti-NGF antibody developed a cataract within 30° day from the beginning of the experiment; all the rats treated with saline solution developed a cataract within 45° day from the beginning of the experiment, whereas only two rats of the group treated with NGF (20%) developed a cataract within 45° day.

10 Studies on the effect of NGF in the form of collyrium for retina pathologies

To evaluate the efficacy of the NGF administration on ocular surface for retina pathologies in a first step experiments disclosed in literature carried out on animal models were repeated using, in addition to intravitreous or retrobulbar administrations, the administration of NGF in the form of collyrium, every two hours, at a concentration of 250 µg/ml in saline balanced solution. In all the experiments both in retinal ischemic and ocular hypertonia damage NGF administered in the form of collyrium exhibited the same activity as when administered by other administration routes.

On the basis of the results obtained from animals a total of 7 patients were treated, three of which suffering from pigmentary retinopathy, two for senile atrophic maculopathy and one for myopic retinopathy. Therapeutic protocol included the instillation of one or two drops of NGF in the form of collyrium at a concentration of 250 µg/ml in balanced saline solution every two hours for 4 weeks. Treatment results were evaluated by objective exam, electroretinogram (ERG), blood flow from central retina artery (evaluated by OBF),

contrast sensitivity, thickness of the layer of nervous fibers (evaluated by OCT), microperimetry and visus.

5 After 4 weeks of treatment all the considered parameters resulted remarkably better; particularly an improvement of ERG, blood flow, contrast sensitivity values and an increase of nervous fibers, microperimetry and visus were detected. Obtained data are summarised in the following Table 4.

Table 4

Effect of treatment with NGF in the form of collyrium on retina pathologies

Pat. No.	Pathology	Age years Sex	Treatment form	Treatment with NGF	ERG ¹⁾	OBF ²⁾	Contrast sensitivity	OCT ³⁾	Microperimetry	Visus
1	Pigmentary retinopathy	35, F	collyrium	4 weeks	++	+	++	+	+	++
2	Pigmentary retinopathy	40, F	collyrium	4 weeks	++	+/-	++	+	+	++
3	Pigmentary retinopathy	32, M	collyrium	4 weeks	+++	++	++	+	++	++++
4	macular foramen	55, F	collyrium	4 weeks	+	+	+	+++	+++	+++
5	senile macular degeneration	70, F	collyrium	4 weeks	+	+/-	+	++	+++	+++
6	senile macular degeneration	73, M	collyrium	4 weeks	+/-	+/-	+	++	++	+
7	miopic retinopathy	26, M	collyrium	4 weeks	+	+	+	++	+++	+++

The values are expressed as improvement with reference to the values before the treatment with NGF: "-" = constant or worsening; "+/-" = improvement < 10 %; "+" = improvement between 11 % and 25 %; "++" = improvement between 26 % and 50 %; "+++" = improvement between 51 % and 75 %; "++++" = improvement higher than 75 %;

¹⁾ ERG = electroretinogram; ²⁾ OBF = blood flow of central retina artery; ³⁾ OCT = thickness of the nervous fiber layer.

Studies on the effect of NGF in the form of collyrium for optic nerve pathologies

To evaluate the efficacy of the NGF administration on ocular surface in retina pathologies in a first step experiments carried out on animal models already disclosed in literature were repeated using, in addition to already disclosed intravitreous or retrobulbar administrations, also the administration of NGF in the form of collyrium, every two hours, at a concentration of 250 µg/ml in saline balanced solution. In all the experiments of crash and ischemic affection of optic nerve NGF administered in the form of collyrium exhibited the same activity as when administered using other administration routes.

On the base of results obtained from animals a total of 7 patients were treated, three of which suffering from low pressure glaucoma, two for retrobulbar neuritis and two for ischemic optic neuritis. Therapeutic protocol included the instillation of one-two drops of NGF in the form of collyrium at a concentration of 200 µg/ml in balanced saline solution every two hours for 4 weeks.

Treatment results were evaluated by objective exam, visual evoked potentials (PEV), blood flow from central retina artery (evaluated by OBF), contrast sensitivity, thickness of the layer of nervous fibers (evaluated by OCT), microperimetry, visual field and visus.

After 4 weeks of treatment all the considered parameters resulted remarkably better; particularly an improvement of PEV, blood flow, contrast sensitivity values and an increase of nervous fibers, microperimetry, visual field and visus were detected. The obtained data are summarised in the following Table 5.

Table 5

Effect of treatment with NGF in the form of collyrium on optic nerve pathologies

Pat. No.	Pathology	Age years Sex	Treatment with NGF	PEV ⁽¹⁾	OBF ⁽²⁾	Contrast sensitivity	OCT ⁽³⁾	Microperimetry	Visual field	Visus
1	normal pres-sure glaucoma	45, F	4 weeks	+++	++	++	++	++	++	++
2	normal pres-sure glaucoma	37, F	4 weeks	++	+	+	++	++	+	+
3	normal pres-sure glaucoma	42, M	4 weeks	+	++	+	++	++	++	++
4	idiopathic optic neuritis	41, M	4 weeks	++	++	+	+	++	+	++
5	idiopathic optic neuritis	38, F	4 weeks	++	++	+	+/-	+	+/-	+
6	ischemic optic neuritis	52, F	4 weeks	++	++	++	+	+/-	+	++
7	ischemic optic neuritis	58, F	4 weeks	++	++	+	++	++	+	++

Values are expressed as improvement with reference to the values before the treatment with NGF: "-"
5 " = constant or worsening; "+/-" = improvement < 19 %;
"+" = improvement between 11 % and 25 %; "++" = improvement between 26 % and 50 %; "+++" = improvement between 51 % and 75 %; "++++" = improvement higher than 75 %;

10 ¹⁾ ERG = electroretinogram; ²⁾ OBF = blood flow of central retina artery; ³⁾ OCT = thickness of the nervous fiber layer.

Studies on the effect of NGF for vitreous body pathologies

15 A balanced saline solution containing 250 µg/ml of NGF was administrated three times a day for 4 weeks to 4 patients affected by myiodesopsia due to the presence of mobile vitreous bodies. After 4 weeks of treatment all the patients recognised symptomatology amelioration.

20 Studies on the effect of NGF for choroidea pathology

To evaluate the effect of external ophthalmic administration of NGF on choroidea pathologies an animal model of autoimmune uveitis, obtained by administration
25 of S retinal antigen to rats, was used. A group of animals every two hours was treated with one drop of NGF in the form of collyrium at a concentration of 200 µg/ml in saline balanced solution. After 4 weeks of treatment the lesions over vitreous body-retina in animals treated
30 with NGF in the form of collyrium were compared to those present in animals treated with saline solution. In all

the animals treated with NGF a reduction of tissues lesions was clearly visible.

The present invention was described with reference to specific embodiments thereof but it to be is intended
5 that variations and modifications can be made by those skilled in the art without departing from the scope thereof.

Claims

1. Use of nerve growth factor (NGF) for the production of an ophthalmic preparation to be administered onto the ocular surface for therapy and/or prophylaxis of intraocular tissue pathologies.

2. Use according to claim 1, wherein said ophthalmic preparation is in form of solution or suspension, ointment, gel or liniment in combination with a pharmaceutically acceptable ophthalmic carrier or in form of ocular erodible insert or polymeric membrane "reservoir" system to be located in the conjunctiva sac or is added to a local bandage together with a therapeutic contact lens.

3. Use according to claims 1 or 2, wherein said ophthalmic preparation is suitable for therapy and/or prophylaxis of sclera, ciliary body, crystalline lens, retina, optic nerve, vitreous body and choroidea pathologies.

4. Use according to claim 3, wherein said pathologies have trophic, post-traumatic, infective, post-surgical, autoimmune, dystrophic, degenerative, post-inflammatory and laser treatment origin.

5. Use according to anyone of claims 1-4, wherein said ophthalmic preparation contains from 1 to 1000 $\mu\text{g/ml}$ of NGF.

6. Use according to claim 5, wherein said ophthalmic preparation is in the form of collyrium and contains from 10 to 500 $\mu\text{g/ml}$ of NGF.

7. Use according to claim 6, wherein said collyrium contains from 200-250 $\mu\text{g/ml}$ of NGF.

8. Use according to anyone of claims 1-7, wherein NGF in said preparation is in association with one or

more of other active principles and/or is conjugated with a carrier molecule.

5 9. Use according to anyone of preceding claims wherein said NGF is of murine or human origin or it is human recombinant NGF.

10 10. Use of nerve growth factor (NGF) for the production of an ophthalmic preparation for therapy and/or prophylaxis of intraocular tissue pathologies, except retina and optic nerve pathologies.

11. Use according to claims 10, wherein said ophthalmic preparation is suitable for therapy and/or prophylaxis of sclera, ciliary body, crystalline lens, vitreous body and choroidea pathologies.

15 12. Use according to claim 11, wherein said pathologies have trophic, post-traumatic, infective, post-surgical, autoimmune, dystrophic, degenerative, post-inflammatory and laser treatment origin.

20 13. Use according to anyone of claims 1-4, wherein said ophthalmic preparation contains from 1 to 1000 µg/ml of NGF.

14. Use of nerve growth factor in therapy for intraocular tissue pathologies according to anyone of claims 1-13, substantially as above described.

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INTERNATIONAL SEARCH REPORT

 Inter. Appl. No.
 PCT/00/00016

 A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 A61K38/18 A61K9/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 48002 A (A. LAMBIASE) 29 October 1998 (1998-10-29) cited in the application claims 8-15	1-14
X	WO 98 10785 A (S. OKAMOTO) 19 March 1998 (1998-03-19) abstract -& EP 0 958 831 A	1-9, 13, 14
X	WO 90 12590 A (STATE OF OREGON, STATE BOARD OF HIGHER EDUCATION...) 1 November 1990 (1990-11-01) claims 1-10	1-14
	-/---	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

15 May 2000

Date of mailing of the international search report

22/05/2000

Name and mailing address of the ISA

 European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
 Fax: (+31-70) 340-3016

Authorized officer

Siatou, E

INTERNATIONAL SEARCH REPORT

Patent Application No.

PCT/IT 00/00016

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 312 208 A (ETHICON INC.) 19 April 1989 (1989-04-19) page 3, line 36 - line 40 page 4, line 7 - line 9 page 5, line 20 - line 29	1-14
X	PATENT ABSTRACTS OF JAPAN vol. 1998, no. 13, 30 November 1998 (1998-11-30) & JP 10 218787 A (OKAMOTO AKIO), 18 August 1998 (1998-08-18) abstract	1-9, 13, 14
X	LAMBIASE ET AL: "Nerve growth factor delays retinal degeneration in C3H mice" CHEMICAL ABSTRACTS + INDEXES, US, AMERICAN CHEMICAL SOCIETY. COLUMBUS, vol. 125, no. 19, 4 November 1996 (1996-11-04), XP002058562 ISSN: 0009-2258 cited in the application & Graefe's Arch. Clin. Exp. Ophthalmol. 1996, 234 (Suppl. 1), S96-S100 abstract	1-4, 14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/JP00/00016

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9848002	A	29-10-1998	IT RM970238 A AU 5135098 A EP 0973872 A	26-10-1998 13-11-1998 26-01-2000
WO 9810785	A	19-03-1998	AU 4220997 A BR 9712824 A CN 1233963 A EP 0958831 A	02-04-1998 21-12-1999 03-11-1999 24-11-1999
WO 9012590	A	01-11-1990	US 5260059 A AU 5551090 A	09-11-1993 16-11-1990
EP 312208	A	19-04-1989	AU 2223588 A GR 88100617 A, B JP 2000112 A MX 169808 B NZ 226171 A PT 88541 A US 5457093 A US 5705485 A US 5427778 A ZA 8806947 A	23-03-1989 22-06-1989 05-01-1990 27-07-1993 26-06-1990 31-07-1989 10-10-1995 06-01-1998 27-06-1995 30-05-1990
JP 10218787	A	18-08-1998	NONE	

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PATENT COOPERATION TREATY

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PCT

From the INTERNATIONAL BUREAU

NOTIFICATION CONCERNING
SUBMISSION OR TRANSMITTAL
OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

BANCHETTI, Marina
Ing. Barzanò & Zanardo Roma S.p.A.
Via Piemonte, 26
I-00187 Roma
ITALIE

Date of mailing (day/month/year) 13 June 2000 (13.06.00)	
Applicant's or agent's file reference PCT24255	IMPORTANT NOTIFICATION
International application No. PCT/IT00/00016	International filing date (day/month/year) 21 January 2000 (21.01.00)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 29 January 1999 (29.01.99)
Applicant ANABASIS S.R.L. et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).
2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.
3. An asterisk(*) appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.
4. The letters "NR" appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, the attention of the applicant is directed to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

<u>Priority date</u>	<u>Priority application No.</u>	<u>Country or regional Office or PCT receiving Office</u>	<u>Date of receipt of priority document</u>
29 Janu 1999 (29.01.99)	RM99A000069	IT	18 May 2000 (18.05.00)

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No. (41-22) 740.14.35	Authorized officer Carlos Naranjo Telephone No. (41-22) 338.83.38
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PATENT COOPERATION TREATY

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INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference PCT24255	FOR FURTHER ACTION		see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
International application No. PCT/IT 00/ 00016	International filing date (day/month/year) 21/01/2000	(Earliest) Priority Date (day/month/year) 29/01/1999	
Applicant ANABASIS S.R.L. et al.			

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of the sequence listing :

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority in written form.

☐ furnished subsequently to this Authority in computer readable form.

☐ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☐ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☐ Certain claims were found unsearchable (See Box I).

3. ☐ Unity of invention is lacking (see Box II).

4. With regard to the title,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the abstract,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the drawing to be published with the abstract is Figure No. _____

☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☐ Non of the figures.

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PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL SEARCH REPORT
OR THE DECLARATION

(PCT Rule 44.1)

To:

BARZANO & ZANARDO ROMA S.P.A.
Attn: Banchetti, Marina
26, Via Piemonte
00187 ROMA
ITALY

Date of mailing
(day/month/year)

22/05/2000

Applicant's or agent's file reference

PCT24255

FOR FURTHER ACTION

See paragraphs 1 and 4 below

International application No.

PCT/IT 00/ 00016

International filing date
(day/month/year)

21/01/2000

Applicant

ANABASIS S.R.L. et al.

1. ☒ The applicant is hereby notified that the International Search Report has been established and is transmitted herewith.

Filing of amendments and statement under Article 19:

The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally 2 months from the date of transmittal of the International Search Report; however, for more details, see the notes on the accompanying sheet.

Where? Directly to the International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland
Facsimile No.: (41-22) 740.14.35

For more detailed instructions, see the notes on the accompanying sheet.

2. ☐ The applicant is hereby notified that no International Search Report will be established and that the declaration under Article 17(2)(a) to that effect is transmitted herewith.

3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:

☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.

☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Further action(s):** The applicant is reminded of the following:

Shortly after 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90bis.1 and 90bis.3, respectively, before the completion of the technical preparations for international publication.

Within 19 months from the priority date, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later).

Within 20 months from the priority date, the applicant must perform the prescribed acts for entry into the national phase before all designated Offices which have not been elected in the demand or in a later election within 19 months from the priority date or could not be elected because they are not bound by Chapter II.

Name and mailing address of the International Searching Authority



European Patent Office, P.B. 5818 Patentlaan 2
NL-2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Chantal Meyer

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The letter must indicate the differences between the claims as filed and the claims as amended. It must, in particular, indicate, in connection with each claim appearing in the international application (it being understood that identical indications concerning several claims may be grouped), whether

- (i) the claim is unchanged;
- (ii) the claim is cancelled;
- (iii) the claim is new;
- (iv) the claim replaces one or more claims as filed;
- (v) the claim is the result of the division of a claim as filed.

The following examples illustrate the manner in which amendments must be explained in the accompanying letter:

1. [Where originally there were 48 claims and after amendment of some claims there are 51]:
"Claims 1 to 29, 31, 32, 34, 35, 37 to 48 replaced by amended claims bearing the same numbers; claims 30, 33 and 36 unchanged; new claims 49 to 51 added."
2. [Where originally there were 15 claims and after amendment of all claims there are 11]:
"Claims 1 to 15 replaced by amended claims 1 to 11."
3. [Where originally there were 14 claims and the amendments consist in cancelling some claims and in adding new claims]:
"Claims 1 to 6 and 14 unchanged; claims 7 to 13 cancelled; new claims 15, 16 and 17 added." or
"Claims 7 to 13 cancelled; new claims 15, 16 and 17 added; all other claims unchanged."
4. [Where various kinds of amendments are made]:
"Claims 1-10 unchanged; claims 11 to 13, 18 and 19 cancelled; claims 14, 15 and 16 replaced by amended claim 14; claim 17 subdivided into amended claims 15, 16 and 17; new claims 20 and 21 added."

"Statement under article 19(1)" (Rule 46.4)

The amendments may be accompanied by a statement explaining the amendments and indicating any impact that such amendments might have on the description and the drawings (which cannot be amended under Article 19(1)).

The statement will be published with the international application and the amended claims.

It must be in the language in which the international application is to be published.

It must be brief, not exceeding 500 words if in English or if translated into English.

It should not be confused with and does not replace the letter indicating the differences between the claims as filed and as amended. It must be filed on a separate sheet and must be identified as such by a heading, preferably by using the words "Statement under Article 19(1)."

It may not contain any disparaging comments on the international search report or the relevance of citations contained in that report. Reference to citations, relevant to a given claim, contained in the international search report may be made only in connection with an amendment of that claim.

Consequence if a demand for international preliminary examination has already been filed

If, at the time of filing any amendments under Article 19, a demand for international preliminary examination has already been submitted, the applicant must preferably, at the same time of filing the amendments with the International Bureau, also file a copy of such amendments with the International Preliminary Examining Authority (see Rule 62.2(a), first sentence).

Consequence with regard to translation of the international application for entry into the national phase

The applicant's attention is drawn to the fact that, where upon entry into the national phase, a translation of the claims as amended under Article 19 may have to be furnished to the designated/elected Offices, instead of, or in addition to, the translation of the claims as filed.

For further details on the requirements of each designated/elected Office, see Volume II of the PCT Applicant's Guide.

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PATENT COOPERATION TREATY

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Assistant Commissioner for Patents
United States Patent and Trademark
Office
Box PCT
Washington, D.C.20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 31 August 2000 (31.08.00)	
International application No. PCT/IT00/00016	Applicant's or agent's file reference PCT24255
International filing date (day/month/year) 21 January 2000 (21.01.00)	Priority date (day/month/year) 29 January 1999 (29.01.99)
Applicant LAMBIASE, Alessandro	

1. The designated Office is hereby notified of its election made:

☒ in the demand filed with the International Preliminary Examining Authority on:
28 July 2000 (28.07.00)

☐ in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was
☐ was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer S. Mafla Telephone No.: (41-22) 338.83.38
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PATENT COOPERATION TREATY

PCT

From the INTERNATIONAL BUREAU

INFORMATION CONCERNING ELECTED
OFFICES NOTIFIED OF THEIR ELECTION

(PCT Rule 61.3)

To:

BANCHETTI, Marina
Ing. Barzanò & Zanardo Roma S.p.A.
Via Piemonte, 26
I-00187 Roma
ITALIE

Date of mailing (day/month/year)
31 August 2000 (31.08.00)

Applicant's or agent's file reference
PCT24255

IMPORTANT INFORMATION

International application No.
PCT/IT00/00016

International filing date (day/month/year)
21 January 2000 (21.01.00)

Priority date (day/month/year)
29 January 1999 (29.01.99)

Applicant
ANABASIS S.R.L. et al

1. The applicant is hereby informed that the International Bureau has, according to Article 31(7), notified each of the following Offices of its election:

AP : GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW

EP : AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE

National : AU, BG, BR, CA, CN, CZ, DE, IL, JP, KP, KR, MN, NO, NZ, PL, RO, RU, SE, SK, US

2. The following Offices have waived the requirement for the notification of their election; the notification will be sent to them by the International Bureau only upon their request:

EA : AM, AZ, BY, KG, KZ, MD, RU, TJ, TM

OA : BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG

National : AE, AL, AM, AT, AZ, BA, BB, BY, CH, CR, CU, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IN, IS, KE, KG, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MW, MX, PT, SD, SG, SI, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW

3. The applicant is reminded that he must enter the "national phase" before the expiration of 30 months from the priority date before each of the Offices listed above. This must be done by paying the national fee(s) and furnishing, if prescribed, a translation of the international application (Article 39(1)(a)), as well as, where applicable, by furnishing a translation of any annexes of the international preliminary examination report (Article 36(3)(b) and Rule 74.1).

Some offices have fixed time limits expiring later than the above-mentioned time limit. For detailed information about the applicable time limits and the acts to be performed upon entry into the national phase before a particular Office, see Volume II of the PCT Applicant's Guide.

The entry into the European regional phase is postponed until 31 months from the priority date for all States designated for the purposes of obtaining a European patent.

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Authorized officer:

S. Maffla

Facsimile No. (41-22) 740.14.35

Telephone No. (41-22) 338.83.38

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PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference

(if desired) (12 characters maximum) PCT24255

Box No. I TITLE OF INVENTION: USE OF NERVE GROWTH FACTOR FOR THERAPY OF INTRAOCULAR TISSUE PATHOLOGIES.

Box No. II APPLICANT

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.
The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

NABASIS s.r.l.
Via delle Robinie, 45
00172 ROMA - ITALY

☐ This person is also inventor

Telephone No.
0335/7046521

Facsimile No.

Teleprinter No.

State (that is, country) of nationality:
ITALY

State (that is, country) of residence:
ITALY

This person is applicant ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.
The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

LAMBIASE Alessandro
Via delle Robinie, 45
00172 ROMA - ITALY

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only (If this check-box is marked, do not fill in below.)

State (that is, country) of nationality:
ITALY

State (that is, country) of residence:
ITALY

This person is applicant ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:

☒ agent

☐ common representative

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)
BANCHETTI Marina - CAPASSO Olga - de SIMONE Domenico - FIORUZZI Maria Augusta - IANNONE Carlo Luigi - TALIERCIO Antonio - ZANARDO Giovanni - ING. BARZANO & ZANARDO ROMA S.p.A. - Via Piemonte 26 - 00187 ROMA - ITALY

Telephone No.
06/4743241

Facsimile No.
06/4870273

Teleprinter No.
625579

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

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Box No. V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) (mark the applicable check-boxes; at least one must be marked):

Regional Patent

<input checked="" type="checkbox"/>	X	AP	ARIPO Patent: GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SL Sierra Leone, SZ Swaziland, UG Uganda, TZ Tanzania, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
-------------------------------------	----------	-----------	---

<input checked="" type="checkbox"/>	X	EA	Eurasian Patent: AM Armenia, AZ Azerbaijan, BY Belarus, KG Kyrgyzstan, KZ Kazakhstan, MD Republic of Moldova, RU Russian Federation, TJ Tajikistan, TM Turkmenistan, and any other State which is a Contracting State of the Eurasian Patent Convention and of the PCT
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<input checked="" type="checkbox"/>	X	EP	European Patent: AT Austria, BE Belgium, CH and LI Switzerland and Liechtenstein, CY Cyprus, DE Germany, DK Denmark, ES Spain, FI Finland, FR France, GB United Kingdom, GR Greece, IE Ireland, IT Italy, LU Luxembourg, MC Monaco, NL Netherlands, PT Portugal, SE Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
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<input checked="" type="checkbox"/>	X	OA	OAPI Patent: BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, GW Guinea-Bissau, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT (if other king of protection or treatment desired, specify on dotted line)
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National Patent (if other king of protection or treatment desired, specify on dotted line):

<input checked="" type="checkbox"/>	X	AE	United Arab Emirates	<input checked="" type="checkbox"/>	LR	Liberia
<input checked="" type="checkbox"/>	X	AL	Albania	<input checked="" type="checkbox"/>	LS	Lesotho
<input checked="" type="checkbox"/>	X	AM	Armenia	<input checked="" type="checkbox"/>	LT	Lithuania
<input checked="" type="checkbox"/>	X	AT	Austria	<input checked="" type="checkbox"/>	LU	Luxembourg
<input checked="" type="checkbox"/>	X	AU	Australia	<input checked="" type="checkbox"/>	LV	Latvia
<input checked="" type="checkbox"/>	X	AZ	Azerbaijan	<input checked="" type="checkbox"/>	MA	Morocco
<input checked="" type="checkbox"/>	X	BA	Bosnia and Herzegovina	<input checked="" type="checkbox"/>	MD	Republic of Moldova
<input checked="" type="checkbox"/>	X	BB	Barbados	<input checked="" type="checkbox"/>	MG	Madagascar
<input checked="" type="checkbox"/>	X	BG	Bulgaria	<input checked="" type="checkbox"/>	MK	The former Yugoslav Republic of Macedonia
<input checked="" type="checkbox"/>	X	BR	Brazil	<input checked="" type="checkbox"/>	MN	Mongolia
<input checked="" type="checkbox"/>	X	BY	Belarus	<input checked="" type="checkbox"/>	MW	Malawi
<input checked="" type="checkbox"/>	X	CA	Canada	<input checked="" type="checkbox"/>	MX	Mexico
<input checked="" type="checkbox"/>	X	CH and LI	Switzerland and Liechtenstein	<input checked="" type="checkbox"/>	NO	Norway
<input checked="" type="checkbox"/>	X	CN	China	<input checked="" type="checkbox"/>	NZ	New Zealand
<input checked="" type="checkbox"/>	X	CU	Cuba	<input checked="" type="checkbox"/>	PL	Poland
<input checked="" type="checkbox"/>	X	CR	Costa Rica	<input checked="" type="checkbox"/>	PT	Portugal
<input checked="" type="checkbox"/>	X	CZ	Czech Republic	<input checked="" type="checkbox"/>	RO	Romania
<input checked="" type="checkbox"/>	X	DE	Germany	<input checked="" type="checkbox"/>	RU	Russian Federation
<input checked="" type="checkbox"/>	X	DK	Denmark	<input checked="" type="checkbox"/>	SD	Sudan
<input checked="" type="checkbox"/>	X	DM	Dominica	<input checked="" type="checkbox"/>	SE	Sweden
<input checked="" type="checkbox"/>	X	EE	Estonia	<input checked="" type="checkbox"/>	SG	Singapore
<input checked="" type="checkbox"/>	X	ES	Spain	<input checked="" type="checkbox"/>	SI	Slovenia
<input checked="" type="checkbox"/>	X	FI	Finland	<input checked="" type="checkbox"/>	SK	Slovakia
<input checked="" type="checkbox"/>	X	GB	United Kingdom	<input checked="" type="checkbox"/>	SL	Sierra Leone
<input checked="" type="checkbox"/>	X	GD	Grenada	<input checked="" type="checkbox"/>	TJ	Tajikistan
<input checked="" type="checkbox"/>	X	GE	Georgia	<input checked="" type="checkbox"/>	TM	Turkmenistan
<input checked="" type="checkbox"/>	X	GH	Ghana	<input checked="" type="checkbox"/>	TR	Turkey
<input checked="" type="checkbox"/>	X	GM	Gambia	<input checked="" type="checkbox"/>	TT	Trinidad and Tobago
<input checked="" type="checkbox"/>	X	HR	Croatia	<input checked="" type="checkbox"/>	TZ	Tanzania
<input checked="" type="checkbox"/>	X	HU	Hungary	<input checked="" type="checkbox"/>	UA	Ukraine
<input checked="" type="checkbox"/>	X	ID	Indonesia	<input checked="" type="checkbox"/>	UG	Uganda
<input checked="" type="checkbox"/>	X	IL	Israel	<input checked="" type="checkbox"/>	US	United States of America
<input checked="" type="checkbox"/>	X	IN	India	<input checked="" type="checkbox"/>	UZ	Uzbekistan
<input checked="" type="checkbox"/>	X	IS	Iceland			
<input checked="" type="checkbox"/>	X	JP	Japan	<input checked="" type="checkbox"/>	VN	Viet Nam
<input checked="" type="checkbox"/>	X	KE	Kenya	<input checked="" type="checkbox"/>	YU	Yugoslavia
<input checked="" type="checkbox"/>	X	KG	Kyrgyzstan	<input checked="" type="checkbox"/>	ZA	South Africa
				<input checked="" type="checkbox"/>	ZW	Zimbabwe
<input checked="" type="checkbox"/>	X	KP	Democratic People's Republic of Korea	Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:		
<input checked="" type="checkbox"/>	X	KR	Republic of Korea			
<input checked="" type="checkbox"/>	X	KZ	Kazakhstan			
<input checked="" type="checkbox"/>	X	LC	Saint Lucia			
<input checked="" type="checkbox"/>	X	LK	Sri Lanka			

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. (Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)

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Box No. VI PRIORITY CLAIM		Further priority claims are indicated in the Supplemental Box.		
Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is:		
		national application: country	regional application:* regional Office	international application: receiving Office
item (1) 29/01/99 29 JANUARY 1999	RM99A000069	ITALY		
item (2)				
item (3)				

☒ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) (only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office) identified above as item(s): (1)

* Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.

Box No. VII INTERNATIONAL SEARCHING AUTHORITY			
Choice of International Searching Authority (ISA) (if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used): ISA /	Request to use results of earlier search; reference to that search (if an earlier search has been carried out by or requested from the International Searching Authority): <div style="display: flex; justify-content: space-between;"> Date (Day/month/year) Number Country (or regional Office) </div>		

Box No. VIII CHECK LIST; LANGUAGE OF FILING	
This international application contains the following number of sheets: request : 3 description (excluding sequence listing part) : 29 claims : 2 abstract : 1 drawings : sequence listing part of description : Total number of sheets : 35	This international application is accompanied by the item(s) marked below: 1. <input checked="" type="checkbox"/> fee calculation sheet 2. <input checked="" type="checkbox"/> separate signed power of attorney 3. <input type="checkbox"/> copy of general power of attorney; reference number, if any: 4. <input type="checkbox"/> statement explaining lack of signature 5. <input type="checkbox"/> priority document(s) identified in Box No. VI as item(s): 6. <input type="checkbox"/> translation of international application into (language): 7. <input type="checkbox"/> separate indications concerning deposited microorganism or other biological material 8. <input type="checkbox"/> nucleotide and/or amino acid sequence listing in computer readable form 9. <input type="checkbox"/> other (specify):
Figure of the drawings which should accompany the abstract:	Language of filing of the international application: ENGLISH

Box No. IX SIGNATURE OF APPLICANT OR AGENT	
Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).	
CAPASSO Olga	

For receiving Office use only

1. Date of actual receipt of the purported international application 3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application: 4. Date of timely receipt of the required corrections under PCT Article 11(2): 5. International Searching Authority (if two or more are competent): ISA /	2. Drawings: <input type="checkbox"/> received: <input type="checkbox"/> not received: 6. <input type="checkbox"/> Transmittal of search copy delayed until search fee is paid.
---	--

For International Bureau use only

Date of receipt of the record copy by the International Bureau:	See Notes to the request form
---	-------------------------------

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PCT**FEE CALCULATION SHEET**

Annex to the Request

For receiving Office use only

International application No.

Date stamp of the receiving Office

Applicant's or agent's
file reference PCT24255

Applicant ANABASIS s.r.l.

CALCULATION OF PRESCRIBED FEES

1. TRANSMITTAL FEE

60.000 T

2. SEARCH FEE

1.829.775 S

International search to be carried out by _____

(If two or more International Searching Authorities are competent in relation to the international application, indicate the name of the Authority which is chosen to carry out the international search.)

INTERNATIONAL FEE**Basic Fee**

The international application contains 35 sheets.

first 30 sheets

791.934 b1

5

x 17.426

=

87.130 b2

remaining sheets

additional amount

Add amounts entered at b1 and b2 and enter total at B

879.064 B

Designation Fees

The international application contains _____ designations.

x

=

1.363.136 D

number of designation fees payable (maximum 10) amount of designation fee

Add amounts entered at B and D and enter total at I

2.242.200 I

(Applicants from certain States are entitled to a reduction of 75% of the international fee. Where the applicant is (or all applicants are) so entitled, the total to be entered at I is 25% of the sum of the amounts entered at B and D.)

4. FEE FOR PRIORITY DOCUMENT (if applicable)

P

J. TOTAL FEES PAYABLE

4.131.975

Add amounts entered at T, S, I and P, and enter total in the TOTAL box

TOTAL

☐ The designation fees are not paid at this time.**MODE OF PAYMENT**☐ authorization to charge
deposit account (see below)☒ bank draft☐ coupons☐ cheque☐ cash☐ other (specify):☐ postal money order☐ revenue stamps**DEPOSIT ACCOUNT AUTHORIZATION** (this mode of payment may not be available at all receiving Offices)The RO/ _____ ☐ is hereby authorized to charge the total fees indicated above to my deposit account.☐ is hereby authorized to charge any deficiency or credit any overpayment in the total fees indicated above to my deposit account.☐ is hereby authorized to charge the fees for preparation and transmittal of the priority document to the International Bureau of WIPO to my deposit account.

Deposit Account No.

Date (day/month/year)

Signature

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PATENT COOPERATION TREATY

g. Vicent
PCT

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

Banchetti, Marina et al.
BARZANO & ZANARDO ROMA S.P.A.
26, Via Piemonte
00187 ROMA
ITALIE

NOTIFICATION OF RECEIPT OF DEMAND BY COMPETENT INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

(PCT Rules 59.3(e) and 61.1(b), first sentence
and Administrative Instructions, Section 601(a))

Date of mailing
(day/month/year)

16. 08. 00

Applicant's or agent's file reference

PCT24255

IMPORTANT NOTIFICATION

International application No.

PCT/IT 00/ 00016

International filing date (day/month/year)

21/01/2000

Priority date (day/month/year)

29/01/1999

Applicant

ANABASIS S.R.L. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority considers the following date as the date of receipt of the demand for international preliminary examination of the international application:

28/07/2000

2. This date of receipt is:

- ☒ the actual date of receipt of the demand by this Authority (Rule 61.1(b)).
☐ the actual date of receipt of the demand on behalf of this Authority (Rule 59.3(e)).
☐ the date on which this Authority has, in response to the invitation to correct defects in the demand (Form PCT/IPEA/404), received the required corrections.

3. ☐ **ATTENTION:** That date of receipt is **AFTER** the expiration of 19 months from the priority date. Consequently, the election(s) made in the demand does (do) not have the effect of postponing the entry into the national phase until 30 months from the priority date (or later in some Offices) (Article 39(1)). Therefore, the acts for entry into the national phase must be performed within 20 months from the priority date (or later in some Offices) (Article 22). For details, see the *PCT Applicant's Guide*, Volume II.

- ☐ (If applicable) This notification confirms the information given by telephone, facsimile transmission or in person on:

4. Only where paragraph 3 applies, a copy of this notification has been sent to the International Bureau.

Name and mailing address of the IPEA/

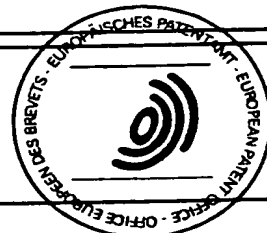


European Patent Office
D-80298 Munich
Tel. (+49-89) 2399-0, Tx: 523656 epmu d
Fax: (+49-89) 2399-4465

Authorized officer

LUOMA M P

Tel. (+49-89) 2399-8929



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PATENT COOPERATION TREATY

PCT

REC'D 16 MAY 2001

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

14

Applicant's or agent's file reference PCT24255		FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/IT00/00016	International filing date (day/month/year) 21/01/2000	Priority date (day/month/year) 29/01/1999
International Patent Classification (IPC) or national classification and IPC A61K38/18		
Applicant ANABASIS S.R.L. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.


2. This REPORT consists of a total of 6 sheets, including this cover sheet.

- ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 33 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand 28/07/2000	Date of completion of this report 14.05.2001
Name and mailing address of the international preliminary examining authority:  European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Didelon, F Telephone No. +49 89 2399 7332



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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/IT00/00016

I. Basis of the report

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):
Description, pages:

1-5,5a,6-9,9a, with telefax of 07/02/2001
10-29

Claims, No.:

1-12 with telefax of 01/02/2001

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
☐ the language of publication of the international application (under Rule 48.3(b)).
☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
☐ filed together with the international application in computer readable form.
☐ furnished subsequently to this Authority in written form.
☐ furnished subsequently to this Authority in computer readable form.
☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been

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**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/IT00/00016

considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	10
	No:	Claims	1-9, 11-12
Inventive step (IS)	Yes:	Claims	10
	No:	Claims	1-9, 11-12
Industrial applicability (IA)	Yes:	Claims	1-12
	No:	Claims	

2. Citations and explanations
see separate sheet

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**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/IT00/00016

Comments on item V:

1. Reference is made to the following documents:

D1: PATENT ABSTRACTS OF JAPAN vol. 1998, no. 13, 30 November 1998 (1998-11-30) & JP 10 218787 A (OKAMOTO AKIO), 18 August 1998 (1998-08-18)

D2: EP-A-0 312 208 (ETHICON INC.) 19 April 1989 (1989-04-19)

D3: WO 90 12590 A (STATE OF OREGON, STATE BOARD OF HIGHER EDUCATION...) 1 November 1990 (1990-11-01)

D4: WO 98 10785 A (S. OKAMOTO) 19 March 1998 (1998-03-19) -& EP 0 958 831 A

D5: WO 98 48002 A (A. LAMBIASE) 29 October 1998 (1998-10-29) cited in the application

D6: LAMBIASE ET AL: 'Nerve growth factor delays retinal degeneration in C3H mice' CHEMICAL ABSTRACTS + INDEXES, US, AMERICAN CHEMICAL SOCIETY. COLUMBUS, vol. 125, no. 19, 4 November 1996 (1996-11-04), XP002058562 ISSN: 0009-2258 cited in the application

1. The amendments received 01.02.2001 with letter dated 31.01.2000 are acknowledged and are considered to meet the requirements of Article 34(2)(b)PCT.
2. Document D3 relates to the treatment by NGF (among other factors) of open-angle glaucoma (due to intraocular pressure), or other ocular diseases, by site-specific administration, like microinjection in the trabecular meshwork.
This differs from the subject-matter of the present claims in that the administration is not made onto the ocular surface.

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Document D5 relates to the use of NGF in the treatment of corneal and conjunctival diseases, in other words of diseases of external tissues of the eye. This differs from the treatment of intraocular tissues pathologies of the present application.

Document D6 relates to a mouse strain presenting retinal degeneration. NGF is shown to inhibit retinal degeneration but the administration route differs from the present application, because intraocular or retroocular injections are used instead of topical application. Thus D6 is not to be regarded as relevant for the subject-matter of the present claims.

3. The present application would not meet the requirements of Article 33(2) PCT because the subject matter of claims 1-9, 11 and 12 does not appear to be novel.

Document D2 discloses the use of NGF in concentrations of 1-500 µg/ml (page 4, lines 7-11) for the treatment of ophthalmic wounds, which can damage intraocular tissues like the anterior chamber of the eye, or the subconjunctive tissue (see page 6, lines 3-11).

This document appears to be prejudicial for the novelty of claims 1-8 and 9, 11 and 12 of the present application.

In addition, documents D1 and D4 from presumably the same author (Okamoto) both relate to the use of NGF in the treatment of optic nerve diseases. In particular, document D4 (EP 0 958 831 A) envisages the use of NGF in concentrations falling within the range considered by the present application. The concentration of NGF can vary between 10 pg/ml (10^{-3} µg/l) and 200 µg/ml (2×10^5 µg/l), as described on page 3, paragraph 0022 (lines 44-45). Although the examples show that the concentration range actually tested is much lower, and that such concentrations are not sufficient for NGF to be present at the site of action, this documents nevertheless teaches to the person skilled in the art that NGF can be used in the same concentration for the treatment of optic nerve diseases, one of the intraocular diseases encompassed by the subject-matter of claims 1 and 3.

In view of this document the subject-matter of claims 1-8 does not appear to be novel.

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**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/IT00/00016

4. The subject-matter of claims 10 is considered as novel over the above-cited documents because only optic nerve diseases and ophtalmic wound of the anterior chamber are considered in these documents.
- Pathologies affecting the sclera, ciliary bodies, crystalline lens, vitreous body and choroidea are not known as being treated by administration of NGF at the surface of the eye, nor is this suggested in the prior art documents. Said claim 10 is therefore considered as involving an inventive activity.

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USE OF NERVE GROWTH FACTOR FOR THERAPY OF INTRAOCULAR
TISSUE PATHOLOGIES

5 The present invention relates to the use of nerve
growth factor for the therapy of intraocular tissue
pathologies. More particularly, the invention relates to
the use of the neurotrophin, named nerve growth factor
(NGF), for the therapeutic treatment of the eye internal
structures, as sclera, choroidea, ciliary bodies,
10 crystalline lens, vitreous body, retina and optic nerve,
by a topical administration over the ocular surface, i.e.
as collyrium or ophthalmic ointment.

The nerve growth factor (NGF) is the chief molecule
of a complex neurotrophin family, and is well known for
its trophic, tropic and differentiating activity on
15 cholinergic neurons of the central nervous system and on
the sympathetic peripheral system. NGF is produced by
various mammalian tissues, included humans, and is
released in the blood stream in greater amounts during
the growth and differentiation of the nervous system.
20 Biological, biochemical and molecular studies carried out
on *in vitro* cellular systems have pointed out high
sequence homology between murine and human NGF.
Furthermore, in humans and other mammals NGF is
25 normally contained both in the cerebrospinalis liquor and
blood stream at concentrations of about 10-15 pg/ml. The
value increases during some inflammatory pathologies
(autoimmune and allergic diseases, etc.), whereas it
decreases in others (diabetes).

30 NGF has been discovered by Prof. Rita Levi-Montal-
cini, at the Zoology Institute of the Washington Univer-

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sity of St. Louis (Levi-Montalcini R., Harvey Lect., 60:217, 1966), and its discovery represented a remarkable advance in the study of the growth and differentiation mechanisms of the nerve cell, as NGF is able to affect

5 the development and preservation of the biological functions of the neurons and their regeneration. In 1986 the Nobel Prize for Medicine and Physiology was awarded to Prof. R. Levi-Montalcini for the discovery of this molecule and the characterization of its biological
10 function both in peripheral and in central nervous system

Various experimental studies both *in vitro* and *in vivo* have demonstrated the physiopathological importance of NGF in preventing neuronal injury of surgical, chemical, mechanical and ischemic origin, thereby making
15 it the ideal candidate for use in the therapy of various pathologies affecting both the peripheral and central nervous systems (Hefti F., J. Neurobiol., 25:1418, 1994; J. Fricker, Lancet, 349:480, 1997). In fact since some years ago clinical tests are being carried out on
20 subjects affected by Parkinson's Disease and Alzheimer's Disease by intracerebral administration of murine NGF (see, for example, Olson L. et al., J. Neural Trans.: Parkinson's Disease and Dementia Section, 4:79, 1992). Results of these experiments confirmed the observations
25 obtained from animal models and pointed out the absence of possible side effects following the administration of murine NGF. This behaviour has been confirmed more recently for recombinant human NGF (Petty B.G. et al., Annals of Neurobiology, 36:244-246, 1994).

30 Studies on the characterization of biological, biochemical, molecular, pre-clinical and clinical effects of NGF almost exclusively have been carried out

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using NGF isolated from submandibular glands of adult rodents; therefore available data concern mostly murine NGF. The biochemical properties of the latter, particularly, have been described in a study published in 1968 (Levi-Montalcini R. and Angeletti P.U., Physiological Reviews, 48:534, 1968).

The NGF contained in murine salivary glands is a 140 kdalton molecular complex, the sedimentation coefficient thereof being 7S, and it is constituted by three sub-units, α , β and γ , the second one of which represents the actual active form. The latter, called β NGF, whose sedimentation coefficient is 2.5S, is usually extracted and purified according to three not very different techniques (Bocchini V., Angeletti P.U., Biochemistry, 64:787-793, 1969; Varon S. et al., Methods in Neurochemistry, 203-229, 1972; Mobley W.C. et al., Molecular Brain Research, 387:53-62, 1986).

The so obtained β NGF is a dimer of ~ 13.000 dalton, consisting of two identical chains of 118 amino acids. Each chain is stabilised by three disulphide bridges, while non-covalent bonds assure the stabilisation of the dimeric structure. The molecule is very stable and is soluble in almost all solvents, both aqueous and oily, maintaining unchanged its biochemical characteristics and biological activity. Further details about the structure, physical and biochemical properties of the molecule are reported in Green, L.A. and Shooter, E.M., Ann. Rev. Neurosci., 3:353, 1980.

Recently the structure of β NGF has been further disclosed by means of crystallographic analysis. The analysis pointed out the presence of three anti-parallel

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filament pairs, having a β -type secondary structure, forming a flat surface along which the two chains join together resulting in the active dimer. On these β NGF chains the presence of four "loop" regions has been showed, wherein many variable amino acids are included. These variable amino acids are probably responsible for receptor recognition specificity.

The biological effect of NGF is mediated by two receptors present on the surface of the corresponding target cells. The existence of various antibodies that selectively inhibit the NGF biological effect has allowed an accurate characterization and modulation of the activity thereof, both in cellular systems and in vivo.

More recently human NGF has been synthesized using genetic engineering techniques (Iwane et al., Biochem. Biophys. Res. Commun., 171:116, 1990) and small amounts of human NGF are commercially available too. However, direct experimentation has shown that the biological activity of human NGF is very low when compared to murine NGF. Furthermore it is to be pointed out that almost all of data available concerning human NGF, both in vivo and in vitro, have been obtained using murine NGF and no undesirable side-effects resulting from the murine origin of the molecule have ever been detected.

Studies carried out on animal models starting in the 90's suggested a possible involvement of NGF in ocular pathologies. As far as the scientific works are concerned, the reports published in the ophthalmic field exclusively refer to the use of NGF in retinal affections and in affections of the optic nerve, i.e. on the nervous tissue.

Particularly, it has been reported that the

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intraocular administration of NGF to animal models is effective in enhancing the survival of retinal ganglion cells following acute retinal ischemia (Siliprandi R. et al., *Inv. Ophthalmol. Vis. Sci.*, 34:3232, 1993) and following optic nerve transection (Carmignoto G. et al., *J. Neurosci.*, 9:1263, 1989). More recently the NGF administration by intravitreous or also retrobulbar injection proved to be effective in a mouse retinal degeneration model, which is similar to human retinitis pigmentosa (Lambiase A. and Aloe L., *Graefe's Arch. Clin. Exp. Ophthalmol.*, 234:S96-S100, 1996), and in a rabbit retinal damage model resulting from ocular hypertension (Lambiase A. et al., *Graefe's Arch. Clin. Exp. Ophthalmol.*, 235:780-785, 1997).

Such experimental studies showed that the local administration of NGF is effective for preventing or at least delaying the death of retinal ganglion cells and photoreceptors resulting from the above pathologies. In addition no side effects during the animal treatments have been reported. However, it is to be pointed out that in all the scientific publications referred to above, NGF is administered to the internal ocular tissue by intravitreous injection or by retrobulbar injection.

In agreement with the foregoing, the PCT patent application WO 90/12590 discloses a method of treating open-angle glaucoma and a method of treating retinal disease, both consisting of providing to the trabecular meshwork of the eye or to the optic cup, respectively, a substance chosen from a wide range of biologically active proteins, among which also NGF is cited. In both cases, an effective amount of the substance must be provided to

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the affected ocular tissue by site specific delivery means, the preferred method of delivery being by microinjection.

5 With specific reference to the disorders affecting the exposed ocular surface, i.e. corneal and conjunctival diseases, EP-A-0312208 discloses gel formulations for use in the treatment of epithelial lesions and epithelial pathologies in general, including lesions and pathologies of the ocular surface. The said formulations contain an
10 active ingredient which may be indiscriminately chosen among the various molecules whose name contains the expression "growth factor". Although the description is exclusively concerned with the epidermal growth factor (EGF) as the preferred active ingredient, and although
15 activity data (*in vitro*) and formulation examples are given only for EGF, other growth factors are mentioned as well, such as FGF (fibroblast growth factor), PDGF (platelet-derived growth factor), TGF- α (transforming growth factor) or the NGF itself. The said growth factors
20 are apparently presented as a family of molecules having equivalent characteristics and biological activity as EGF. As a matter of fact, at the current state of the knowledge, it is undisputed that the said growth factors have different specific targets and that they often have
25 conflicting effects, so that they are not considered as biologically equivalent to each other.

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The use of NGF for external ophthalmic administration, for example in the form of collyrium or ointment, is actually described in the PCT patent application WO 98/48002, having the same inventor as the instant application. The experimental work therein reported proves that topically administered NGF is suitable for a successful treatment of ocular surface pathologies (i.e., affecting cornea and conjunctiva) both of acquired and congenital type and, particularly, of various dystrophic or neurodystrophic pathologies for which therapeutic treatments did not exist previously. The discovery of the presence of NGF and of its high affinity receptor (TrkA, tyrosine kinase A) in corneal tissue, made by immunohistochemical techniques, was the preliminary step for such innovative result. Evidently the expression of the NGF high affinity receptor is an essential prerequisite for NGF to exert its therapeutic activity.

In the frame of the studies which led to the present invention it has been found, by both immunohistochemical and immunofluorescence techniques (Lambiase et al., J. Allergy Clin. Immunol., 100:408-414, 1997) and, in addition, by biomolecular techniques for the *in situ* identification of the NGF mRNA (Micera A. et al., Archives Italiennes de Biologie, 133:131-142, 1995), that all cells of the sclera, crystalline anterior capsule, ciliary body epithelium, the optic nerve fibers, the retinal ganglion cells, the retinal pigmented epithelium cells and some choroidea cells not only express the high affinity receptor for NGF but are also able to produce this neurotrophin (not yet published data). These experimental data result in various implications. On the one hand NGF, released from cells of various ocular

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tissues, should exhibit a trophic and physiopathological activity in all the ocular regenerative mechanisms; on the other hand, various pathologies of trophic, degenerative or immune type should recognise the failed release of NGF as a fundamental etiologic step.

Furthermore, as the effects observed after the administration of exogenous NGF are present at almost physiological concentrations (in the order of about a few micrograms), it is conceivable that in some ocular affections the reduction of local NGF levels under the threshold value suitable to assure the tissue integrity can be a possible physiopathogenetic mechanism. Such a pathogenetic hypothesis is confirmed by some published data concerning the effects of NGF deprivation. The latter induces, both *in vitro* and *in vivo*, the death of various cell populations and the exacerbation of tissue damages of chemical, physical, infective or degenerative type (Aloe L., *Int. J. Devl. Neuroscience*, volume 5(4), 1987; Lambiase A. and Aloe L., cited above; Lambiase et al., Graefe's Arch. Clin. Exp. Ophthalmol., 1997, cited above).

Although the above results allow to hypothesise a therapeutic activity of NGF also on ocular structures and tissues different from those already reported in the literature, and specifically on sclera, ciliary bodies, crystalline, vitreous body and choroidea, there is the problem of an easy administration of the active principle to the involved tissues. Contrary to the case considered in the PCT patent application WO 98/48002, referring to corneal and conjunctival pathologies, in this case tissues within the eyeball are involved.

The possibility of administering an ophthalmic therapeutic agent by the external ophthalmic route, i.e.

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in the form of collyrium or ointment, represents a remarkable benefit in comparison with the administration through the topical parenteral route, e.g. by retrobulbar or intravitreal injection. In fact the use of these latter techniques involves the risk for various complications, reported in literature, such as the ocular bulb perforation, infections, haemorrhages and lesions of anatomical structures during injection. Such complications can occur even more frequently during the treatment of chronic pathologies, and can lead to the unfeasibility of the therapy due to the inversion of the risk/benefit ratio.

It has now surprisingly been found that by administering NGF in the form of collyrium, an increase of the neurotrophin levels in all ocular tissues, including those internal to the ocular bulb, is obtained. As it will be illustrated in detail in the following experimental report, the passage of NGF from the ocular surface, where it is administered, to internal ocular tissues, has been shown using both an autoradiographic method (Levi-Montalcini, R. and Aloe L., Proc. Natl. Sci. USA 82:7111-7115, 1985), and an immunoenzymatic assay (Bracci-Laudiero, L. et al., Neurosci. Lett., 147:9-12, 1992). The application of the latter method on rabbits treated by conjunctival instillation of a NGF-containing saline solution has caused, one hour after the administration, an increase of NGF concentration in all the examined ocular tissues. The NGF level is reduced to initial levels after 6-8 hours. This effect allows NGF to perform its therapeutic activity also in tissues not directly involved by a superficial administration. This aspect is innovative not only with reference to the ophthalmic pathologies for which till now the NGF

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therapeutic activity had not even been hypothesized, but also for the retinal and optic nerve pathologies, wherein the potential activity of NGF has been already reported, but it was not possible to administer the drug in a ready and safe way without risks and drawbacks for the patient.

The only known publications wherein a topical ophthalmic administration of NGF is disclosed, for the therapy of glaucoma and optic nerve affections, are the Japanese patent application JP 10 218787 and the EP-A-0958831, having closely related subject-matter, both designing Okamoto as the inventor. Apparently, NGF or a derivative thereof, in admixture with, or as an alternative to another neurotrophin, brain-derived neurotrophic factor (BDNF) or a derivative thereof, are proposed for the above therapeutic purposes for administration to the eye by any route, including the external application as eye drops or by means of a medicated contact lens. In spite of the fact that the description of EP-A-0958831 recites extremely wide ranges of concentrations for the active neurotrophin in the medicinal preparation by which it is to be administered, the only actual data available, given in the examples, recite NGF concentrations, in an ophthalmic solution, of 0.04 and 0.02 µg/ml. Any person skilled in the art who tried to carry out the above teachings in an *in vivo* test, thereby administering such concentrations of NGF by the external ophthalmic route, would not be able to detect any effect on the clinical conditions concerned. Also any experimental test on animals would confirm that NGF, administered on the eye surface as eye drops at the concentrations specified above, would not pass in a detectable amount from the ocular surface to the internal tissues.

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Accordingly, the present invention specifically provides, according to a first aspect thereof, the use of nerve growth factor (NGF) for the production of an ophthalmic preparation for administration over the ocular surface for the therapy and/or the prophylaxis of pathologies affecting the internal tissues of the eye, wherein said ophthalmic preparation contains from 10 to 500 µg/ml of NGF. Specifically said NGF-containing ophthalmic preparation is in the form of a solution or a suspension, an ointment, a gel or a cream in a pharmaceutically acceptable carrier, which is tolerated by the eye and compatible with the active principle. It is also possible to conceive particular routes of ophthalmic administration for delayed release, as ocular erodible inserts, or polymeric membrane "reservoir" systems to be located in the conjunctival sac. Alternatively NGF, or a preparation containing it, can be added to a local bandage together with a therapeutic contact lens.

As already pointed out said ophthalmic preparation is suitable for the therapy and/or the prophylaxis of pathologies affecting the sclera, ciliary bodies, crystalline lens, retina, optic nerve, vitreous body and choroidea, said affections having trophic, post-traumatic, infective, post-surgical, autoimmune, dystrophic, degenerative or post-inflammatory origin, or being originated by laser treatment. As it will be demonstrated by the experimental data reported below, the external topical administration of NGF proved, among other things, to be able to repair scleral lesions of traumatic or immune

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origin, to cause an increase of aqueous humour production, restoring the intraocular pressure in pathologies characterised by hypotonia and resulting in bulbar phthisis, and to prevent and delay the formation and progression of crystalline lens opacity (cataract).
5 As to the retinal pathologies, the NGF administration by application over the ocular surface induces an increase of nervous fiber thickness, a survival of retinal ganglion cells, photoreceptors, and pigmented epithelium
10 during degenerative, ischemic, traumatic pathologies and when damages from ocular hypertonia are present. As to the optic nerve, the effects obtained are an improvement of visual evoked potentials (VEP), of visual field and of the survival of nervous fibers when traumatic, ischemic,
15 pressor and degenerative pathologies occur. Finally, as to choroidea the NGF administration by external ophthalmic application causes a reduction of choroidea inflammatory processes and reduces the number of mobile vitreous bodies. It is to be pointed out that many of
20 these disorders are hardly therapeutically treated, or they lack any effective treatment.

The possibility that nerve growth factor could exhibit a biological activity on internal tissues of the ocular bulb following an external local administration
25 was hardly predictable mainly considering that, as pointed out before, NGF is a quite big molecule (26,800 dalton) with a complex structure. In order that an exogenous molecule can exert its activity on deep ocular tissues, it is necessary that, once it has been instilled
30 over the eye surface, the molecule pass through the lacrimal layer, the cornea, the aqueous humour and the vitreous body so to be distributed within all the

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tissues. According to the current practice no molecules (particularly antibiotics or cortisone molecules) which are able to reach the crystalline lens, vitreous body and retina at therapeutically effective concentrations are presently available. For the above reasons in all the known studies on the utilisation of NGF for ocular pathologies, only the intraocular administration route was used.

In effect NGF, although having a complex structure and high molecular weight, includes both hydrophilic and hydrophobic groups which allow it to pass through the homologous (lipid and hydrophilic) anatomical barriers. Furthermore it is a basic characteristic of NGF that once it has reached the target organs, also at very low but yet biologically active concentrations, it is able to stimulate tissue to produce endogenously the NGF. The presence of an endogenous fraction of NGF is clearly suggested by experimental results on the passage of NGF through tissues. These results furthermore show that a concentration gradient is not maintained from the external surface to deeper eye tissues, as it would be conceivable in the presence of a simple diffusion mechanism through the tissues.

In order to produce the preparation according to the present invention, suitable procedures for the NGF extraction and purification are reported in the previously cited references. The technique according to Bocchini and Angeletti, herein briefly reported, has been used for the experiments of the present invention. Submandibular glands of adult male mice are collected in a sterile way and tissues thereof are homogenised, centrifuged and dialysed; then the obtained suspension is

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passed through subsequent cellulose columns, whereon NGF is adsorbed. Then NGF is eluted with a buffer containing 0.4 M sodium chloride. The obtained samples are analysed spectrophotometrically at a 289nm wavelength to identify the NGF containing fractions. These fractions are dialysed and the NGF is lyophilised in a sterile way and stored at -20°C in freezer.

A medicament according to the invention suitable for administration onto the ocular surface contains, alone or optionally in association with one or more other active principles, from 10 to 500 µg/ml of NGF. In the case the product is in the form of an aqueous solution (collyrium), the concentration of NGF is preferably between 200 and 250 µg/ml. A specific formulation suitable in the form of collyrium contains, for example, 200 µg/ml of NGF in physiological solution containing 0.9% of sodium chloride, or in balanced saline solution (BSS^R); in both circumstances the solution is isotonic with the tear fluid and therefore well tolerable by the eye. However it is also possible the use of hypotonic solutions.

The NGF contained in the saline solution can be present alone or in association with other biologically active molecules, and/or conjugated with carrier molecules (as, for example, transferrin). In order to further enhance its passage through ocular surface, other excipients selected from those conventionally used according to pharmaceutical techniques, for example to buffer the solutions or suspensions, to stabilise the active principle and make the preparation well tolerable can be added. Specifically buffers should keep pH between 4 and 8. For example the above reported sodium chloride

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solution can be buffered using any of the buffers well known in the pharmaceutically field as suitable for ophthalmic use, among which phosphate or trizma (tri-hydroxymethyl-aminomethane) buffers, so as to have a physiological pH, i.e. 7.0-7.4, maintaining simultaneously a physiological osmolarity (295-305 mOsm/l).

The tolerability can be further enhanced using excipients like polysorbate 80 (or Tween 80), dextran, polyethylene glycol (for example PEG 400) and like. The formulation can contain also viscosity-enhancing agents like hyaluronic acid, methylcellulose, polyvinylalcohol, polyvinylpyrrolidone and others, in order to enhance the ocular bioavailability, stability and tolerability of the active principle. The ocular bioavailability of NGF can be further enhanced by using compounds that ameliorate the corneal permeation of the drug as, for example, dimethylsulfoxide, taurocholates, membrane phospholipids and various surfactant agents suitable for ophthalmic use. In addition, to prevent contamination, a preservative agent having antimicrobial activity can be added to the formulation.

Agents like carboxymethylcellulose or the like can be added to products to be administered in the form of suspension. If it is desired to use the formulation in the form of ointment, gel or ophthalmic cream, the NGF carrier could be polyethyleneglycol, polyacrylate, polyethyleneoxide, fatty acid and alcohol or lanolin, paraffin and similar products.

As already pointed out the therapeutic activity of nerve growth factor on ocular tissues other than the superficial ones (cornea and conjunctiva), as well as on

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the retina, and on the optic nerve, has not been previously disclosed, neither when it is administered by intraocular injection or when it is administered in formulations in the form of collyrium or ointment.

5 Therefore the present invention further provides, according to another specific aspect thereof, the use of nerve growth factor (NGF) for the production of an ophthalmic preparation for the therapy and/or the prophylaxis of pathologies affecting the internal tissues
10 of the eye, except retina and optic nerve pathologies, whatever the administration route is.

The concentration of NGF in the preparation is preferably between 10 and 500 µg/ml of NGF and all the conventional formulation procedures well known in the
15 field can be used and particularly those previously reported with reference to the ophthalmic formulations for external administration.

Some experimental results, obtained within the frame of the present invention, including clinical data
20 concerning therapeutic applications on humans, are reported below merely for exemplary purposes.

Studies on the passage of NGF through the ocular tissues

In a first set of tests to study the passage of NGF intraocularly from the external surface over which it was
25 administered, the above mentioned autoradiographic method has been used for a group of six rabbits. Each of the animals was administered with one collyrium drop (50 µl) containing 10 µg of I¹²⁵ labeled NGF (concentration: 200 µg/ml) by instillation in the conjunctival fornix.

30 Murine NGF purified according to the previously described method and subsequently conjugated to Na-I¹²⁵

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(Amersham Italia, IMS30, 1mCi) according to the chloramine T method (Lapack PA. Exp. Neurol. 124:1620, 1993) has been used. The amount of labeled NGF has been determined by chromatography using a Sephadex G-25 column. The amount of the I¹²⁵ labeled product collectible by precipitation was between 90% and 95%, showing that most of the radioactive product was bonded to NGF. The specific activity of NGF-I¹²⁵ was between 1 and 1.5 Ci/ μ mol.

Two hours following the administration of the labeled NGF the animals were sacrificed and eyes enucleated and fixed in 4% paraformaldehyde over 48 hours. Then samples, after incubation in 30% sucrose over 24 hours, were cut with a cryostat to 15 μ m thick sections. Sections were mounted on histology gelatinous slides, immersed in photographic emulsion (Ilford K2) and incubated over 4 weeks at 4°C. The sections were successively dehydrated using ethanol, mounted on DPX after treatment with xylene and examined with Zeiss optical microscope.

This experiment showed that labeled NGF, after its administration over the ocular surface, was able to penetrate into the eye and bond with cells of various tissues contained in the posterior segment and crystalline lens inducing the expression of the specific receptor.

In a second set of tests, using the above described immunoenzymatic method, the quantitative levels of NGF in various ocular tissues after the administration by instillation of a drop of murine NGF in the conjunctiva fornix were determined. In all 24 rabbits were used, six thereof were sacrificed immediately to determine the

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baseline values of NGF concentration in various ocular tissues. Remaining animals were sacrificed after 1 (6 rabbits), 2 (6 rabbits) and 8 hours (6 rabbits) following the administration of the collyrium.

5 In all the cases the eyes were enucleated and the different tissues (cornea, sclera, aqueous, iris, crystalline lens, retina, choroidea, optic nerve) were sectioned. The tissues were weighed, sonicated (using Braun B Sonicator) in a buffered protein matrix
10 containing protease inhibitors (extraction buffer). The thus obtained homogenate was centrifuged ($\times 10000$ rpm for 20 minutes) and the supernatant was used to determine the levels on NGF by immunoenzymatic method (ELISA). This technique is extremely sensitive and NGF specific, and it
15 is able to detect concentrations up to 5 pg/ml. Goat anti-NGF polyclonal antibody, diluted in 0.05 M carbonate buffer, pH 9.6, was used as first antibody. As control, for the determination of unspecific signal, purified goat immunoglobulins were used.

20 Solutions containing primary antibody and control immunoglobulins were placed in parallel on polystyrene 96-well plates. Then the plates were incubated for 12 hours at room temperature and then the unspecific sites were blocked using a solution containing carbonate buffer
25 plus 1% BSA. Further to plate washings with 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 0.5% gelatine, and 0.1% Triton X-100, NGF samples and standard solutions were suitably diluted with 50 mM Tris-HCl, pH 7.2, 400 mM NaCl, 4 mM EDTA, 0.2 mM PMSE, 0.2 mM benzethonium chloride, 2 mM
30 b nzimidine, 40 U/ml aprotinin, 0.05% sodium azide, 2 % BSA and 0.5 % gelatine. After triplicat distributions of standard solutions and samples of NGF in an amount of 50

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µm/well, the plates were incubated with the secondary antibody: 4 mU/well of anti-β-galactosidase (Boehringer Mannheim, Germany) for 2 hours at 37°C. Then, after the washings, 100 µl/well of a solution containing 4 mg of β-galactosil-chlorophenol red (Boehringer Mannheim Germany)/ml of 100 mM HEPES, 150 mM NaCl, mM MgCl₂, 0,1% sodium azide and 1% BSA solution were distributed.

After the incubation of the chromogen for a period of two hours at 37°C the optical density at wavelength of 575 nm was determined using an ELISA reader (Dynatech). The concentration values of NGF standards and samples were calculated after subtraction of background values due to unspecific bonds. Data reported as pg/ml or pg/g are referred to fresh weighed tissue. The results, summarized in the following Table 1, show that: after one hour from the collyrium administration the NGF concentration values are increased in all the intraocular tissues, these values are maintained high, although reduced, after 2 hours, and after 8 hours they are again the same as the baseline ones.

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Table 1

NGF concentrations in various ocular tissues after NGF
administration in the form of collyrium
(NGF pg/g of tissue)

HRS	SCLERA	CHOROIDEA	RETINA	OPTIC NERVE	CRYSTALLINE LENS	VITREOUS BODY
0	100 \pm 50	960 \pm 400	83 \pm 50	83 \pm 50	100 \pm 15	10 \pm 4
1	1414 \pm 30	2800 \pm 700	484 \pm 70	1195 \pm 180	200 \pm 30	73 \pm 12
2	694 \pm 150	1813 \pm 900	322 \pm 100	342 \pm 115	150 \pm 20	20 \pm 5
3	200 \pm 100	100 \pm 500	150 \pm 70	130 \pm 100	110 \pm 20	10 \pm 5

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Studies on the effect of NGF administration in the form
of collyrium for scleral pathologies

Presently no therapeutic treatments effective to
induce reparations for both traumatic and immune or
infective scleral lesions are known. In the case of
autoimmune pathologies the formation of malacic sclera
zones (scleromalacia) occurs which tend progressively to
enlarge and become deeper with possible bulb perforation.
Surgical treatment is the unique usable therapy and it
includes the coating of damaged or malacic zone with a
layer of human stored sclera or other biocompatible human
tissues. However, in the case of immune affections,
recidivations of the scleral pathology often occur.

In the studies in connection with the present
invention the effect of external administration in the
form of collyrium of murine NGF (2.5S), at a
concentration of 250 μ g/ml in balanced saline solution,
was evaluated for 4 cases of scleral lesions, 2 of which

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post-traumatic and 2 scleromalacic due to autoimmune diseases (rheumatoid arthritis, AR and systemic lupus erythematosus, respectively). Therapeutic protocol included the daily instillation of one or two drops of preparation in the following way: during the first two days every two hours, six times a day up to the second day from the complete sclera reparation and four times a day during the following fifteen days. The therapy, once interrupted, should be immediately restarted if initial signals or symptoms of recidivations of scleral pathology are present.

All the patients within two weeks from the beginning of the treatment with NGF showed clear signals of recovery. None thereof showed occurrence of local or systemic side effects during or after the treatment. Obtained data are summarised in the following table.

Table 2

Effect of treatment with NGF in the form of collyrium for scleral pathologies

Pat. No.	Pathology	Age years Sex	Occurrence	Extension	NGF Treatment	Outcome	Follow up
1	perforating trauma	35, F	4 days	4 mm	21 days	recovery	8 months
2	perforating trauma	42, M	5 days	6 mm	25 days	recovery	6 months
3	scleromacia in AR	55, F	30 days	5 mm	20 days	recovery	10 months
4	scleromacia in LES	42, M	25 days	4 mm	17 days	recovery	8 months

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Studies on the effect of NGF administration in the form of collyrium for the production of aqueous humour

The effect of topical administration of NGF on the production of aqueous humour was determined first on a set of 6 rabbits with normal intraocular pressure. Using a tomography based method including a probe in anterior chamber of eye which is able to evaluate the modifications in the production of aqueous humour, it was recognised that the administration of NGF in the form of collyrium every two hours at a concentration of 200 µg/ml, in balanced saline solution, induces a five-fold increase in the production of aqueous humour. Such an increase is maintained during all the period of treatment.

On the base of the results obtained on animal model three patients with remarkable ocular hypotonia were treated, two of which showed hypotonia following surgical treatments (2 eyes) and the other as a result of a recurrent chronic uveitis. Due to very low intraocular pressure values (< 4 mm Hg), rapidly medical conditions were degenerating to bulb phthisis. The therapeutic protocol included the instillation of one or two drops of NGF preparation (200 µg/ml) in balanced saline solution every two hours until a successful clinical outcome.

All the treated patients exhibited clear symptoms of recovery within two weeks from the beginning of NGF treatment, the intraocular pressure values being again between 8 and 12 mm Hg within 4 weeks. No patient showed the occurrence of local or systemic side effects during the treatment or the following period. Obtained data are summarised in the following table.

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Table 3

Effect of the administration of NGF in the form of collyrium on production of aqueous humour

Pat. No.	Pathology	Age years Sex	Occurrence	NGF Treatment	Outcome	Follow up
1	vitrectomy	40, M	30 days	21 days	9 mm Hg	7 months
2	vitrectomy	53, F	25 days	25 days	10 mm Hg	11 months
3	chronic uveitis	45, F	40 days	20 days	12 mm Hg	10 months

5 Studies on the effect of NGF treatment in the form of collyrium for the cataract prevention

Because it has been recognised that cells of the crystalline lens capsule express the receptor with high affinity for NGF and simultaneously produce this neurotrophin, it was studied whether variations of local values of NGF resulted in formation of crystalline lens opacity (cataract, a process usually related to senescence phenomena, diabetes, steroid treatment, traumas or physical stresses) and whether the external administration of NGF could prevent the formation or progression thereof.

To demonstrate the activity of NGF firstly a model of *in vitro* formation of cataract was used. In the study 18 crystalline lenses from adult rats were collected and incubated in a xilose containing medium. Then 6 crystalline lenses were treated by the addition to the medium of amounts of murine NGF variable between 1 and 300 pg/ml, 6 crystalline lenses were treated by the

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addition of amounts of anti-NGF antibody between 500 and 1000 µg and the remaining were left untreated as control. After 48 hours from the beginning of the culture it was clear that 6 crystalline lenses treated with anti-NGF antibody exhibited almost full cataract, whereas 6 control crystalline lens exhibited cortical cataract with poor involvement of nucleus of crystalline lens. The remaining 6, treated with NGF, exhibited only rare opacity traces, the best response being obtained with NGF concentration of about 200 pg/ml in culture medium.

To confirm the in vivo NGF activity in preventing the cataract occurrence a cataractogenesis animal model involving a diet including 30% glycerol was used. All the animals (100%) subjected to this diet exhibit a cataract within the 44th day. A group comprising ten animals was treated by three daily administrations of NGF in the form of collyrium at a concentration of 200 µg/ml in balanced saline solution, a second group again comprising ten animals was subjected to a treatment with anti-NGF antibodies injected in the anterior camera and the last group of animals was treated with saline solution in drops and was used as control.

All the rats of the group treated with anti-NGF antibody developed a cataract within the 30th day from the beginning of the experiment; all the rats treated with saline solution developed a cataract within the 45th day from the beginning of the experiment, whereas only two rats of the group treated with NGF (20%) developed a cataract within the 45th day.

Studies on the effect of NGF in the form of collyrium for retinal pathologies

To evaluate the efficacy of the NGF administration

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on ocular surface for retinal pathologies in a first step experiments disclosed in literature carried out on animal models were repeated using, in addition to intravitreous or retrobulbar administrations, the administration of NGF in the form of collyrium, every two hours, at a concentration of 250 µg/ml in saline balanced solution. In all the experiments both in retinal ischemic and ocular hypertonia damage NGF administered in the form of collyrium exhibited the same activity as when administered by other administration routes.

On the basis of the results obtained from animals a total of 7 patients were treated, three of which suffering from retinitis pigmentosa, one from macular foramen, two for senile atrophic maculopathy and one for myopic retinopathy. The therapeutic protocol included the instillation of one or two drops of NGF in the form of collyrium at a concentration of 250 µg/ml in balanced saline solution every two hours for 4 weeks. Treatment results were evaluated by objective examination, electroretinogram (ERG), blood flow from central retina artery (evaluated by OBF), contrast sensitivity, thickness of the layer of nervous fibers (evaluated by OCT), microperimetry and visus.

After 4 weeks of treatment all the considered parameters resulted remarkably better; particularly an improvement of ERG, blood flow, contrast sensitivity values and an increase of nervous fibers, microperimetry and visus were detected. Obtained data are summarised in the following Table 4.

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Table 4

Effect of treatment with NGF in the form of collyrium on retinal pathologies

Pat. No.	Pathology	Age years Sex	Treatment form	Treatment with NGF	ERG ¹⁾	OBF ²⁾	Contrast sensitivity	OCT ³⁾	Microperimetry	Visus
1	Retinitis pigmentosa	35, F	collyrium	4 weeks	++	+	++	+	+	++
2	Retinitis pigmentosa	40, F	collyrium	4 weeks	++	+/-	++	+	+	++
3	Retinitis pigmentosa	32, M	collyrium	4 weeks	+++	++	++	+	++	+++
4	macular foramen	55, F	collyrium	4 weeks	+	+	+	+++	+++	+++
5	senile macular degeneration	70, F	collyrium	4 weeks	+	+/-	+	++	+++	+++
6	senile macular degeneration	73, M	collyrium	4 weeks	+/-	+/-	+	++	++	+
7	mdopic retinopathy	26, M	collyrium	4 weeks	+	+	+	++	+++	+++

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The values are expressed as improvement with reference to the values before the treatment with NGF: "-" = constant or worsening; "+/-" = improvement < 10 %; "+" = improvement between 11 % and 25 %; "++" = improvement between 26 % and 50 %; "+++" = improvement between 51 % and 75 %; "++++" = improvement higher than 75 %;

¹⁾ ERG = electroretinogram; ²⁾ OBF = blood flow of central retinal artery; ³⁾ OCT = thickness of the nervous fiber layer.

Studies on the effect of NGF in the form of collyrium for optic nerve pathologies

To evaluate the efficacy of the administration of NGF on the ocular surface in retinal pathologies in a first step experiments carried out on animal models already disclosed in literature were repeated using, in addition to already disclosed intravitreous or retrobulbar administrations, also the administration of NGF in the form of collyrium, every two hours, at a concentration of 250 µg/ml in saline balanced solution. In all the experiments of crash and ischemic injury of optic nerve the NGF administered in the form of collyrium exhibited the same activity as when administered using other administration routes.

On the base of results obtained from animals a total of 7 patients were treated, three of which suffering from low pressure glaucoma, two from retrobulbar neuritis and two from ischemic optic neuritis. The therapeutic protocol included the instillation of one-two drops of NGF in the form of collyrium at a concentration of 200 µg/ml in balanced saline solution every two hours for 4 weeks. Treatment results were evaluated by objective

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5 examination, visual evoked potentials (VEP), blood flow from central retinal artery (evaluated by OBF), contrast sensitivity, thickness of the layer of nervous fibers (evaluated by OCT), microperimetry, visual field and visus.

10 After 4 weeks of treatment all the considered parameters resulted remarkably better; particularly an improvement of VEP, blood flow, contrast sensitivity values and an increase of nervous fibers, microperimetry, visual field and visus were detected. The obtained data are summarised in the following Table 5.

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Table 5

Effect of treatment with NGF in the form of collyrium on optic nerve pathologies

Pat. No.	Pathology	Age years Sex	Treatment with NGF	VEP ¹⁾	OBP ²⁾	Contrast sensitivity	OCT ³⁾	Microperimetry	Visual field	Visus
1	normal pres-sure glaucoma	45, F	4 weeks	+++	++	++	++	++	++	++
2	normal pres-sure glaucoma	37, F	4 weeks	++	+	+	++	++	+	+
3	normal pres-sure glaucoma	42, M	4 weeks	+	++	+	++	++	++	++
4	idiopathic optic neuritis	41, M	4 weeks	++	++	+	+	++	+	++
5	idiopathic optic neuritis	36, F	4 weeks	++	++	+	+/-	+	+/-	+
6	ischemic optic neuritis	52, F	4 weeks	++	++	++	+	+/-	+	++
7	ischemic optic neuritis	58, F	4 weeks	++	++	+	++	++	+	++

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Values are expressed as improvement with reference to the values before the treatment with NGF: "-" = constant or worsening; "+/-" = improvement < 19 %; "+" = improvement between 11 % and 25 %; "++" = improvement between 26 % and 50 %; "+++" = improvement between 51 % and 75 %; "++++" = improvement higher than 75 %;

¹⁾ ERG = electroretinogram; ²⁾ OBF = blood flow of central retinal artery; ³⁾ OCT = thickness of the nervous fiber layer.

Studies on the effect of NGF for vitreous body pathologies

A balanced saline solution containing 250 µg/ml of NGF was administrated three times a day for 4 weeks to 4 patients affected by myiodesopsia due to the presence of mobile vitreous bodies. After 4 weeks of treatment all the patients recognised a symptomatology amelioration.

Studies on the effect of NGF for choroideal pathologies

To evaluate the effect of external ophthalmic administration of NGF on choroideal pathologies an animal model of autoimmune uveitis, obtained by administration of S retinal antigen to rats, was used. A group of animals was treated every two hours with one drop of NGF in the form of collyrium at a concentration of 200 µg/ml in saline balanced solution. After 4 weeks of treatment the lesions over vitreous body-retina in animals treated with NGF in the form of collyrium were compared to those present in animals treated with saline solution. In all the animals treated with NGF a reduction of tissues lesions was clearly visible.

The present invention was described with reference

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to specific embodiments thereof but it to be is intended
that variations and modifications can be made by those
skilled in the art without departing from the scope
thereof as defined in the appended claims.

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Claims

1. Use of nerve growth factor (NGF) for the production of an ophthalmic preparation for administration onto the ocular surface for the therapy and/or the prophylaxis of pathologies affecting the internal tissues of the eye, wherein said ophthalmic preparation contains from 10 to 500 µg/ml of NGF.

2. Use according to claim 1, wherein said ophthalmic preparation is in the form of a solution or a suspension, an ointment, a gel or a cream in a pharmaceutically acceptable ophthalmic carrier or in the form of an ocular erodible insert or a polymeric membrane "reservoir" system to be placed in the conjunctival sac or it is added to a local bandage together with a therapeutic contact lens.

3. Use according to claims 1 or 2, wherein said ophthalmic preparation is suitable for the therapy and/or the prophylaxis of pathologies affecting the sclera, ciliary bodies, crystalline lens, retina, optic nerve, vitreous body and choroidea.

4. Use according to claim 3, wherein said pathologies have trophic, post-traumatic, infective, post-surgical, autoimmune, dystrophic, degenerative or post-inflammatory origin, or are originated by laser treatment.

5. Use according to any one of claims 1-4, wherein said ophthalmic preparation is in the form of an ophthalmic solution.

6. Use according to claim 5, wherein said ophthalmic solution contains from 200-250 µg/ml of NGF.

7. Use according to anyone of claims 1-6, wherein the NGF in said preparation is in association with one or

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more other active ingredients and/or it is conjugated with a carrier molecule.

5 8. Use according to anyone of the preceding claims wherein said NGF is of murine or of human origin, or it is human recombinant NGF.

10 9. Use of nerve growth factor (NGF) for the production of an ophthalmic preparation for the therapy and/or the prophylaxis of pathologies affecting the internal tissues of the eye, except retinal pathologies and pathologies affecting the optic nerve.

15 10. Use according to claim 9, wherein said ophthalmic preparation is suitable for the therapy and/or the prophylaxis of pathologies affecting the sclera, ciliary bodies, crystalline lens, vitreous body and choroidea.

20 11. Use according to claim 10, wherein said pathologies have trophic, post-traumatic, infective, post-surgical, autoimmune, dystrophic, degenerative, or post-inflammatory origin, or are originated by laser treatment.

12. Use according to anyone of claims 9-11, wherein said ophthalmic preparation contains from 200 to 250 µg/ml of NGF.

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PATENT COOPERATION TREATY

PCT

NOTICE INFORMING THE APPLICANT OF THE
COMMUNICATION OF THE INTERNATIONAL
APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

From the INTERNATIONAL BUREAU

To:

BANCHETTI, Marina
Ing. Barzanò & Zanardo Roma S.p.A.
Via Piemonte, 26
I-00187 Roma
ITALIE

Date of mailing (day/month/year)

03 August 2000 (03.08.00)

Applicant's or agent's file reference

PCT24255

IMPORTANT NOTICE

International application No.

PCT/IT00/00016

International filing date (day/month/year)

21 January 2000 (21.01.00)

Priority date (day/month/year)

29 January 1999 (29.01.99)

Applicant

ANABASIS S.R.L. et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:

AU,JP,KP,KR,US

In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:

AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CN,CR,CU,CZ,DE,DK,DM,EA,EE,EP,ES,FI,GB,GD,
GE,GH,GM,HR,HU,ID,IL,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,MW,MX,NO,
NZ,OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,ZA,ZW

The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on 03 August 2000 (03.08.00) under No. WO 00/44396

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, a demand for international preliminary examination must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the national phase, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

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